(54) Title: SELECTIVE TARGETING OF TUMOR VASCULATURE USING ANTIBODY MOLECULES

(57) Abstract: The present invention relates to selectively targeting tumoral vasculature in vivo using a human recombinant scFv, L19, to the angiogenesis marker ED-B domain of fibronectin. In preferred embodiments, a complete human IgG1 is employed having the variable regions of L19. In other embodiments is employed a mini-immunoglobulin generated by fusing the scFv L19 to the constant CH4 domain of a secretory IgE isoform that naturally contains a cysteine in its COOH terminal and which forms a covalently linked dimer. Different in vivo behaviour of the antibody formats is exploitable for different diagnostic and/or therapeutic purposes, depending on clinical needs and disease. The antibody molecules may be labelled as described.

SELECTIVE TARGETING OF TUMOR VASCULATURE
USING ANTIBODY MOLECULES

The present invention relates to targeting of tumor
vasculature using antibody molecules. In particular, the
invention relates to use of antibody molecules that bind ED-B
of fibronectin, and which are of demonstrated usefulness in
tumor targeting. In different embodiments of the present
invention, antibody molecules are employed in different
molecular formats. In certain embodiments the antibody
molecules comprise human IgG1. In other embodiments the
antibody molecules are mini-immunoglobulins, such as are
generated by fusing an scFv antibody molecule to the constant
CH4 domain of a secretory IgE isoform that naturally contains
a cysteine in its COOH terminal which forms a covalently
linked dimer. Blood clearance rate, *in vivo* stability and
other advantageous properties are employed in different
aspects and embodiments of the invention, e.g. in tumor
targeting. The different *in vivo* behavior of different
antibody molecule formats may be exploited for different
diagnostic and/or therapeutic purposes, depending on clinical
needs and disease.

Despite their enormous potential as therapeutic agents,
monoclonal antibodies (mAbs) of non-human origin have
performed poorly in clinical trials as a result of their
immunogenicity (1 Shawlert et al., 1985; 2 Miller et al.,
1983), poor pharmacokinetic properties (3 Hakimi et al., 1991;
4 Stephens et al., 1995) and inefficiency in recruiting
effector functions (5 Riechmann et al., 1988; 6 Junghens et
al., 1990). The recent prospect of isolating human antibody
fragments from phage display libraries (7 McCafferty et al.,
1990; 8 Lowman et al., 1991; for reviews see 9 Nilsonn et al.,

2000 and 10 Winter et al., 1994) transcends these problems, revitalizing studies and rekindling hopes of using these reagents to treat major diseases. Indeed, these molecules should serve as ideal building blocks for novel diagnostic and therapeutic tools (11 Reichert, 2001; 12 Huls et al., 1999). Furthermore, these antibodies can be "matured" to reach affinities in the picomolar range (13 Pini et al., 1998), at least desirable, if not necessary, for their clinical use.

Clinical applications of human antibody fragments for the selective delivery of diagnostic or therapeutic agents nonetheless require highly specific targets. In the case of tumors, the most popular targets are cell-surface antigens, which are usually neither abundant nor stable. Nevertheless, during tumor progression, the microenvironment surrounding tumor cells undergoes extensive modification that generates a "tumoral environment" which represents a target for antibody-based tumor therapy (14 Neri and Zardi, 1998). In fact, the concept that the altered tumor microenvironment is itself a carcinogen that can be targeted is increasingly gaining consensus. Molecules that are able to effectively deliver therapeutic agents to the tumor microenvironment thus represent promising and important new tools for cancer therapy (15 Bissel, 2001; 14 Neri and Zardi, 1998).

Fibronectin is an extracellular matrix (ECM) component that is widely expressed in a variety of normal tissues and body fluids. Different FN isoforms can be generated by the alternative splicing of the FN pre-mRNA, a process that is modulated by cytokines and extracellular pH (16 Balza et al. , 1988; 17 Carnemolla et al., 1989; 18 Borsi et al., 1990; 19 Borsi et al., 1995). The complete type III repeat ED-B, also known as the extratype III repeat B (EIIIB), may be entirely

included or omitted in the FN molecule (20 Zardi et al.,
1987). ED-B is highly conserved in different species, having
100% homology in all mammalians thus far studied (human, rat,
mouse, dog) and 96% homology with a similar domain in chicken.
The FN isoform containing ED-B (B-FN) is undetectable
immunohistochemically in normal adult tissues, with the
exception of tissues undergoing physiological remodelling
(e.g., endometrium and ovary) and during wound healing (17
Carnemolla et al., 1989; 21 ffrench-Constant, et al., 1989).
By contrast, its expression in tumors and fetal tissues is
high (17 Carnemolla et al, 1989). Furthermore, it has been
demonstrated that B-FN is a marker of angiogenesis (22
Castellani et al., 1994) and that endothelial cells invading
tumor tissues migrate along ECM fibers containing B-FN (23
Tarli et al. 1999).

Selective targeting of tumoral vasculature has been described
using a human recombinant antibody, scFv(L19) (13 Pini et al.,
98), specific for the B-FN isoform (24 Carnemolla et al.,
1996; 23 Tarli et al., 99; 25 Viti et al., 99; 26 Neri et al.,
97; 27 Demartis et al., 2001). The antibody may be used in
both in vivo diagnostic (immunoscintigraphy) and therapeutic
approaches entailing the selective delivery of therapeutic
radionuclides or toxic agents to tumoral vasculature. In
addition, Birchler et al. (28 1999) showed that scFv(L19),
chemically coupled to a photosensitizer, selectively
accumulates in the newly formed blood vessels of the
angiogenic rabbit cornea model and, after irradiation with
near infrared light, mediates complete and selective occlusion
of ocular neovasculature.

More recently, Nilsson et al. (29 2001) reported that the
immunoconjugate of scFv(L19) with the extracellular domain of

tissue factor mediates selective infarction in different types
of murine tumor models. Furthermore, fusion proteins of
scFv(L19) and IL-2 or IL-12 have shown the enhanced
therapeutic efficacy of these two cytokines (30 Halin et
al.,submitted; 31 Carnemolla et al., 2002). See also
WO01/62298 for use of fusions in treatment of lesions of
pathological angiogenesis, including tumors. Finally, since
L19 reacts equally well with mouse and human ED-B, it can be
used for both pre-clinical and clinical studies.

See also PCT/GB97/01412, PCT/EP99/03210, PCT/EP01/02062 and
PCT/IB01/00382.

Different antibody formats have shown diverse behavior in
terms of *in vivo* stability, clearance and performance in tumor
targeting (32 Wu et al., 2000). A mini-immunoglobulin or
small immunoprotein (SIP) is described in (33 Li et al.,
1997).

The present invention is based on preparation of,
characterization of and investigation of the *in vivo*
biodistribution of L19 human antibody molecules in different
formats, namely, scFv, mini-immunoglobulin and complete IgG1.

Brief Description of the Figures

Figure 1 shows models illustrating the structures of different
proteins. A: Model of the domain structure of a FN subunit.
The protein sequences undergoing alternative splicing are
indicated in grey. As indicated, the epitope of the
recombinant antibody L19 is localized within the repeat ED-B.
B - D: Schemes of the constructs used to express,
respectively, L19 (scFv) (B); L19-SIP (C); and L19-IgG1/κ.

Figure 2 shows growth curves of SK-MEL-28 tumor in nude mice
(triangles) and of F9 tumor in 129 mouse strain (circles). The
volume (mm^3) is plotted versus time (days). Each data point is
5   the average of six mice $\pm$ SD.

Figure 3 shows the results of size exclusion chromatography on
the different L19 formats. In panels A, B and C are shown
size exclusion chromatography (Superdex 200) profiles of the
10   L19 formats scFv, mini-immunoglobulin and IgG1, respectively,
after radioiodination. Panels D, E and F show size exclusion
chromatography (Superdex 200) profiles of plasma at the
indicated times after i.v. injection of the radioiodinated L19
formats, scFv, mini-immunoglobulin and IgG1, respectively. No
15   changes in the curve profiles of L19-SIP or L19-IgG1 were
detected when loading plasma at different times after
injection, while 3h after L19(scFv)2 injection a second peak
of higher molecular mass was observed.

20   Figure 4 shows results of biodistribution experiments in SK-
MEL-28 tumor-bearing mice using different radioiodinated L19
antibody molecule formats. The variations of the %ID/g in the
tumor (Figure 4A) and in the blood (Figure 4B) at the
indicated times after i.v. injection are reported. In Figure
25   4C the tumor-blood ratios of the %ID/g are plotted. The curves
of L19(scFv) are indicated by diamonds, of L19 mini-
immunoglobulin by squares and of L19 IgG1 by triangles.

Figure 5 shows results of biodistribution experiments in F9
30   tumor-bearing mice using radioiodinated L19(scFv) (squares)
and L19 mini-immunoglobulin (diamonds). The variations of the
%ID/g in the tumor (A) and in the blood (B), at the indicated
different times after i.v. injection are reported

In one aspect, the present invention provides a specific
binding member which binds human ED-B of fibronectin and which
comprises the L19 VH domain and a VL domain, optionally the
5   L19 VL domain, and wherein the specific binding member
comprises a mini-immunoglobulin comprising said antibody VH
domain and antibody VL domain fused to ε_{s2}-CH4 and dimerized or
comprises a whole IgG1 antibody molecule.

10   The L19 VH domain and L19 VL domain sequences are set out in
Pini et al. (1998) *J. Biol. Chem.* 273: 21769-21776, those
sequences being fully incorporated herein by reference to Pini
et al. as if set out here.

15   Generally, a VH domain is paired with a VL domain to provide
an antibody antigen binding site. In a preferred embodiment,
the L19 VH domain is paired with the L19 VL domain, so that an
antibody antigen binding site is formed comprising both the
L19 VH and VL domains. In other embodiments, the L19 VH is
20   paired with a VL domain other than the L19 VL. Light-chain
promiscuity is well established in the art.

One or more CDRs may be taken from the L19 VH or VL domain and
incorporated into a suitable framework. This is discussed
25   further below. L19 VH CDR's 1, 2 and 3 are shown in SEQ ID
NO.'s 1, 2, and 3, respectively. L19 VL CDR's 1, 2 and 3 are
shown in SEQ ID NO.'s 1, 2 and 3, respectively.

Variants of the VH and VL domains and CDRs of which the
30   sequences are set out herein and which can be employed in
specific binding members for ED-B can be obtained by means of
methods of sequence alteration or mutation and screening.

Variable domain amino acid sequence variants of any of the VH
and VL domains whose sequences are specifically disclosed
herein may be employed in accordance with the present
invention, as discussed. Particular variants may include one

5   or more amino acid sequence alterations (addition, deletion,
substitution and/or insertion of an amino acid residue), maybe
less than about 20 alterations, less than about 15
alterations, less than about 10 alterations or less than about
5 alterations, 4, 3, 2 or 1. Alterations may be made in one

10   or more framework regions and/or one or more CDR's.

A specific binding member according to the invention may be
one which competes for binding to antigen with a specific
binding member which both binds ED-B and comprises an antigen-

15   binding site formed of the L19 VH domain and L19 VL domain.
Competition between binding members may be assayed easily *in
vitro*, for example using ELISA and/or by tagging a specific
reporter molecule to one binding member which can be detected
in the presence of other untagged binding member(s), to enable

20   identification of specific binding members which bind the same
epitope or an overlapping epitope.

Thus, further aspects of the present invention employ a
specific binding member comprising a human antibody antigen-

25   binding site which competes with L19 for binding to ED-B.

A specific binding member according to the present invention
may bind ED-B with at least the affinity of L19, binding
affinity of different specific binding members being compared

30   under appropriate conditions.

In addition to antibody sequences, a specific binding member
according to the present invention may comprise other amino

acids, e.g. forming a peptide or polypeptide, such as a folded
domain, or to impart to the molecule another functional
characteristic in addition to ability to bind antigen.
Specific binding members of the invention may carry a
5   detectable label, or may be conjugated to a toxin or enzyme
(e.g. via a peptidyl bond or linker).

In treatment of disorders or lesions of pathological
angiogenesis, a specific binding member of the invention may
10   be conjugated to a toxic molecule, for instance a biocidal or
cytotoxic molecule that may be selected from interleukin-2
(IL-2), doxorubicin, interleukin-12 (IL-12), Interferon-γ (IFN-
γ), Tumor Necrosis Factor α (TNFα) and tissue factor
(preferably truncated tissue factor, e.g. to residues 1-219).
15   See e.g. WO01/62298.

In further aspects, the invention provides an isolated nucleic
acid which comprises a sequence encoding a specific binding
member according to the present invention, and methods of
20   preparing a specific binding member which comprise expressing
said nucleic acid under conditions to bring about production
of said specific binding member and recovering it.

Specific binding members according to the invention may be
25   used in a method of treatment or diagnosis of the human or
animal body, such as a method of treatment (which may include
prophylactic treatment) of a disease or disorder in a human
patient which comprises administering to said patient an
effective amount of a specific binding member of the
30   invention. Conditions treatable in accordance with the
present invention include tumors, especially solid tumors, and
other lesions of pathological angiogenesis, including,

rheumatoid arthritis, diabetic retinopathy, age-related
macular degeneration, and angiomas.

5   A yet further aspect provides a method of producing a specific
       binding member of the invention, the method comprising causing
       expression from encoding nucleic acid. Such a method may
       comprise culturing host cells under conditions for production
       of said specific binding member.

10   A method of production may comprise a step of isolation and/or
       purification of the product.

       A method of production may comprise formulating the product
       into a composition including at least one additional
15   component, such as a pharmaceutically acceptable excipient.

       These and other aspects of the invention are described in
       further detail below.

20   *TERMINOLOGY*

       Specific binding member
       This describes a member of a pair of molecules which have
       binding specificity for one another. The members of a
25   specific binding pair may be naturally derived or wholly or
       partially synthetically produced. One member of the pair of
       molecules has an area on its surface, or a cavity, which
       specifically binds to and is therefore complementary to a
       particular spatial and polar organisation of the other member
30   of the pair of molecules. Thus the members of the pair have
       the property of binding specifically to each other. Examples
       of types of specific binding pairs are antigen-antibody,
       biotin-avidin, hormone-hormone receptor, receptor-ligand,

enzyme-substrate.   This application is concerned with antigen-antibody type reactions.

Antibody molecule

5   This describes an immunoglobulin whether natural or partly or wholly synthetically produced.   The term also covers any polypeptide or protein comprising an antibody binding domain. Antibody fragments which comprise an antigen binding domain are such as Fab, scFv, Fv, dAb, Fd; and diabodies.   The

10   present invention is concerned with whole IgG1 antibody molecules and mini-immunoglobulins comprising ε_{s2}-CH4 as disclosed.

Techniques of recombinant DNA technology may be used to

15   produce from an initial antibody molecule other antibody molecules which retain the specificity of the original antibody molecule.   Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to

20   the constant regions, or constant regions plus framework regions, of a different immunoglobulin.   See, for instance, EP-A-184187, GB 2188638A or EP-A-239400.

As antibodies can be modified in a number of ways, the term

25   "antibody molecule" should be construed as covering any specific binding member or substance having an antibody antigen-binding domain with the required specificity.   Thus, this term covers antibody fragments and derivatives, including any polypeptide comprising an immunoglobulin antigen-binding

30   domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are

therefore included. Cloning and expression of chimeric
antibodies are described in EP-A-0120694 and EP-A-0125023.

Antigen binding domain

This describes the part of an antibody molecule which
comprises the area which specifically binds to and is
complementary to part or all of an antigen. Where an antigen
is large, an antibody may only bind to a particular part of
the antigen, which part is termed an epitope. An antigen
binding domain may be provided by one or more antibody
variable domains (e.g. a so-called Fd antibody fragment
consisting of a VH domain). Preferably, an antigen binding
domain comprises an antibody light chain variable region (VL)
and an antibody heavy chain variable region (VH).

Specific

This may be used to refer to the situation in which one member
of a specific binding pair will not show any significant
binding to molecules other than its specific binding
partner(s). The term is also applicable where e.g. an antigen
binding domain is specific for a particular epitope which is
carried by a number of antigens, in which case the specific
binding member carrying the antigen binding domain will be
able to bind to the various antigens carrying the epitope.

Comprise

This is generally used in the sense of include, that is to say
permitting the presence of one or more features or components.

Isolated

This refers to the state in which specific binding members of
the invention, or nucleic acid encoding such binding members,
will generally be in accordance with the present invention.

Members and nucleic acid will be free or substantially free of
material with which they are naturally associated such as
other polypeptides or nucleic acids with which they are found
in their natural environment, or the environment in which they

5   are prepared (e.g. cell culture) when such preparation is by
recombinant DNA technology practised *in vitro* or *in vivo*.
Members and nucleic acid may be formulated with diluents or
adjuvants and still for practical purposes be isolated - for
example the members will normally be mixed with gelatin or

10   other carriers if used to coat microtitre plates for use in
immunoassays, or will be mixed with pharmaceutically
acceptable carriers or diluents when used in diagnosis or
therapy. Specific binding members may be glycosylated, either
naturally or by systems of heterologous eukaryotic cells (e.g.

15   CHO or NSO (ECACC 85110503) cells, or they may be (for example
if produced by expression in a prokaryotic cell)
unglycosylated.

By "substantially as set out" it is meant that the relevant

20   CDR or VH or VL domain of the invention will be either
identical or highly similar to the specified regions of which
the sequence is set out herein. By "highly similar" it is
contemplated that from 1 to 5, preferably from 1 to 4 such as
1 to 3 or 1 or 2, or 3 or 4, substitutions may be made in the

25   CDR and/or VH or VL domain.

The structure for carrying a CDR of the invention will
generally be of an antibody heavy or light chain sequence or
substantial portion thereof in which the CDR is located at a

30   location corresponding to the CDR of naturally occurring VH
and VL antibody variable domains encoded by rearranged
immunoglobulin genes. The structures and locations of
immunoglobulin variable domains may be determined by reference

to (Kabat, E.A. et al, Sequences of Proteins of Immunological
Interest. 5th Edition. US Department of Health and Human
Services. 1991, and updates thereof, now available on the
Internet (http://immuno.bme.nwu.edu or find "Kabat" using any
search engine).

Preferably, a CDR amino acid sequence substantially as set out
herein is carried as a CDR in a human variable domain or a
substantial portion thereof. The L19 VH CDR3 and/or L19 VL
CDR3 sequences substantially as set out herein may be used in
preferred embodiments of the present invention and it is
preferred that each of these is carried as a CDR3 in a human
heavy or light chain variable domain, as the case may be, or a
substantial portion thereof.

A substantial portion of an immunoglobulin variable domain
will comprise at least the three CDR regions, together with
their intervening framework regions. Preferably, the portion
will also include at least about 50% of either or both of the
first and fourth framework regions, the 50% being the C-
terminal 50% of the first framework region and the N-terminal
50% of the fourth framework region. Additional residues at
the N-terminal or C-terminal end of the substantial part of
the variable domain may be those not normally associated with
naturally occurring variable domain regions. For example,
construction of specific binding members of the present
invention made by recombinant DNA techniques may result in the
introduction of N- or C-terminal residues encoded by linkers
introduced to facilitate cloning or other manipulation steps.
Other manipulation steps include the introduction of linkers
to join variable domains of the invention to further protein
sequences including immunoglobulin heavy chains, other

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variable domains or protein labels as discussed in more
details below.

In an IgG1 antibody molecule according to the present
invention, VL domains may be attached at the C-terminal end to
antibody light chain constant domains including human Cκ or Cλ
chains, preferably Cκ chains.

Specific binding members of the invention may be labelled with
a detectable or functional label. Detectable labels are
described below and include radiolabels such as radioisotopes
of Technetium, Indium, Yttrium, Copper, Lutetium or Rhenium,
in particular ^{94m}Tc, ^{99m}Tc, ^{186}Re, ^{188}Re, ^{111}In, ^{86}Y, ^{88}Y, ^{177}Lu, ^{64}Cu
and ^{67}Cu, which may be attached to antibodies of the invention
using conventional chemistry known in the art of antibody
imaging as described herein.

Labels also include enzyme labels such as horseradish
peroxidase. Labels further include chemical moieties such as
biotin which may be detected via binding to a specific cognate
detectable moiety, e.g. labelled avidin.

The specific binding members (L19-SIP) disclosed herein are
particularly well suited for radiolabeling with isotopes such as
^{94m}Tc, ^{99m}Tc, ^{186}Re, ^{188}Re, ^{203}Pb, ^{67}Ga, ^{68}Ga, ^{43}Sc, ^{47}Sc, ^{110m}In, ^{111}In,
^{97}Ru, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{68}Cu, ^{86}Y, ^{88}Y, ^{90}Y, ^{121}Sn, ^{161}Tb, ^{153}Sm, ^{166}Ho,
^{105}Rh, ^{177}Lu, ^{72}Lu and ^{18}F, and subsequent use in radio-diagnosis
and radiotherapy. ^{99m}Tc is a particularly preferred radioisotope
for labelling, and a suitable protocol is described in the
experimental section below.

To radiolabel the specific binding members directly, the
cysteine bridged molecules are first reduced by an appropriate

reducing agent e.g. stannous(II)chloride, Tris(2-
carboxyethyl)phosphine (TCEP) generating free cysteine SH-groups
that can react with isotopes e.g. Tc or Re. In this particular
procedure, the permetalates obtained from an instant generator
5   system are reduced by a reducing agent e.g. stannous(II)chloride
in the presence of an auxillary ligand e.g. sodium tartrate and
the API (details are provided below in the experimental
section).

10   Indirect labeling with e.g. indium, yttrium, lanthanides or
technetium and rhenium may be performed by pre-conjugating a
chelating ligand, preferably derived from ethylene diamine
tetraacetic acid (EDTA), diethylene triamine pentaacetic acid
(DTPA), cyclohexyl 1,2-diamine tetraacetic acid (CDTA),
15   ethyleneglycol-O,O´-bis(2-aminoethyl)-N,N,N´,N´-diacetic acid
(HBED), triethylene tetraamine hexaacetic acid (TTHA), 1,4,7,10-
tetraazacyclododecane-N,N`,N```-tetraacetic acid (DOTA), 1,4,7-
triazacyclononane-N,N`,N``-triacetic acid (NOTA), 1,4,8,11-
tetraazacyclotetradecane-N,N`,N``,N```-tetraacetic acid (TETA),
20   mercaptoacetyl diglycine (MAG$_2$), mercaptoacetyl triglycine
(MAG$_3$), mercaptoacetyl glycyl cysteine (MAGC), cysteinyl glycyl
cysteine (CGC) to either amine or thiol groups of the specific
binding member. The chelating ligands possess a suitable
coupling group e.g. active esters, maleimides, thiocarbamates or
25   α-halogenated acetamide moieties. For conjugating chelating
ligands to amine groups e.g. ε-NH$_2$-groups of lysine residues
previous reduction of the L-19-SIP compound is not required.

Specific binding members of the present invention are designed
30   to be used in methods of diagnosis or treatment in human or
animal subjects, preferably human.

Accordingly, further aspects of the invention provide methods
of treatment comprising administration of a specific binding
member as provided, pharmaceutical compositions comprising
such a specific binding member, and use of such a specific

5   binding member in the manufacture of a medicament for
administration, for example in a method of making a medicament
or pharmaceutical composition comprising formulating the
specific binding member with a pharmaceutically acceptable
excipient.

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Clinical indications in which a specific binding member of the
invention may be used to provide therapeutic benefit include
tumors such as any solid tumor, also other lesions of
pathological angiogenesis, including rheumatoid arthritis,

15   diabetic retinopathy, age-related macular degeneration, and
angiomas.

Specific binding members according to the invention may be
used in a method of treatment of the human or animal body,

20   such as a method of treatment (which may include prophylactic
treatment) of a disease or disorder in a human patient which
comprises administering to said patient an effective amount of
a specific binding member of the invention. Conditions
treatable in accordance with the present invention are

25   discussed elsewhere herein.

Accordingly, further aspects of the invention provide methods
of treatment comprising administration of a specific binding
member as provided, pharmaceutical compositions comprising

30   such a specific binding member, and use of such a specific
binding member in the manufacture of a medicament for
administration, for example in a method of making a medicament
or pharmaceutical composition comprising formulating the

specific binding member with a pharmaceutically acceptable
excipient.

In accordance with the present invention, compositions
provided may be administered to individuals. Administration
is preferably in a "therapeutically effective amount", this
being sufficient to show benefit to a patient. Such benefit
may be at least amelioration of at least one symptom. The
actual amount administered, and rate and time-course of
administration, will depend on the nature and severity of what
is being treated. Prescription of treatment, e.g. decisions
on dosage etc, is within the responsibility of general
practitioners and other medical doctors. Appropriate doses of
antibody are well known in the art; see Ledermann J.A. et al.
(1991) Int J. Cancer 47: 659-664; Bagshawe K.D. et al. (1991)
Antibody, Immunoconjugates and Radiopharmaceuticals 4: 915-
922.

A composition may be administered alone or in combination with
other treatments, either simultaneously or sequentially
dependent upon the condition to be treated.

Specific binding members of the present invention, including
those comprising an antibody antigen-binding domain, may be
administered to a patient in need of treatment via any
suitable route, usually by injection into the bloodstream
and/or directly into the site to be treated, e.g. tumor. The
precise dose will depend upon a number of factors, the route
of treatment, the size and location of the area to be treated
(e.g. tumor), the precise nature of the antibody (e.g. whole
IgG1 antibody molecule, mini-immunoglobulin molecule), and the
nature of any detectable label or other molecule attached to

the antibody molecule. A typical antibody dose will be in the range 10-50 mg.

This is a dose for a single treatment of an adult patient, which may be proportionally adjusted for children and infants, and also adjusted for other antibody formats in proportion to molecular weight. Treatments may be repeated at daily, twice-weekly, weekly or monthly intervals, at the discretion of the physician.

Specific binding members of the present invention will usually be administered in the form of a pharmaceutical composition, which may comprise at least one component in addition to the specific binding member.

Thus pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous.

For intravenous, injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's

Injection. Preservatives, stabilisers, buffers, antioxidants
and/or other additives may be included, as required.

A composition may be administered alone or in combination with
other treatments, either simultaneously or sequentially
dependent upon the condition to be treated. Other treatments
may include the administration of suitable doses of pain
relief drugs such as non-steroidal anti-inflammatory drugs
(e.g. aspirin, paracetamol, ibuprofen or ketoprofen) or
opiates such as morphine, or anti-emetics.

The present invention provides a method comprising causing or
allowing binding of a specific binding member as provided
herein to ED-B. As noted, such binding may take place *in
vivo*, e.g. following administration of a specific binding
member, or nucleic acid encoding a specific binding member, or
it may take place *in vitro*, for example in ELISA, Western
blotting, immunocytochemistry, immuno-precipitation or
affinity chromatography.

The amount of binding of specific binding member to ED-B may
be determined. Quantitation may be related to the amount of
the antigen in a test sample, which may be of diagnostic
interest, which may be of diagnostic interest.

The reactivities of antibodies on a sample may be determined
by any appropriate means. Radioimmunoassay (RIA) is one
possibility. Radioactive labelled antigen is mixed with
unlabelled antigen (the test sample) and allowed to bind to
the antibody. Bound antigen is physically separated from
unbound antigen and the amount of radioactive antigen bound to
the antibody determined. The more antigen there is in the
test sample the less radioactive antigen will bind to the

antibody. A competitive binding assay may also be used with non-radioactive antigen, using antigen or an analogue linked to a reporter molecule. The reporter molecule may be a fluorochrome, phosphor or laser dye with spectrally isolated

5   absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

10   Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or

15   otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral

20   absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

25   The signals generated by individual antibody-reporter conjugates may be used to derive quantifiable absolute or relative data of the relevant antibody binding in samples (normal and test).

30   The present invention further extends to a specific binding member which competes for binding to ED-B with any specific binding member which both binds the antigen and comprises a V domain including a CDR with amino acid substantially as set

out herein, preferably a VH domain comprising VH CDR3 of SEQ
ID NO. 3. Competition between binding members may be assayed
easily *in vitro*, for example by tagging a specific reporter
molecule to one binding member which can be detected in the
presence of other untagged binding member(s), to enable
identification of specific binding members which bind the same
epitope or an overlapping epitope. Competition may be
determined for example using the ELISA as described in
Carnemolla et al. (24 1996).

The present invention further provides an isolated nucleic
acid encoding a specific binding member of the present
invention. Nucleic acid may be DNA or RNA.

The present invention also provides constructs in the form of
plasmids, vectors, transcription or expression cassettes which
comprise at least one polynucleotide as above.

The present invention also provides a recombinant host cell
which comprises one or more constructs as above. A nucleic
acid encoding a specific binding member as provided itself
forms an aspect of the present invention, as does a method of
production of the encoded product, which method comprises
expression from encoding nucleic acid therefor. Expression
may conveniently be achieved by culturing under appropriate
conditions recombinant host cells containing the nucleic acid.
Following production by expression a specific binding member
may be isolated and/or purified using any suitable technique,
then used as appropriate.

Specific binding members and encoding nucleic acid molecules
and vectors according to the present invention may be provided
isolated and/or purified, e.g. from their natural environment,

in substantially pure or homogeneous form, or, in the case of
nucleic acid, free or substantially free of nucleic acid or
genes origin other than the sequence encoding a polypeptide
with the required function. Nucleic acid according to the
present invention may comprise DNA or RNA and may be wholly or
partially synthetic. Reference to a nucleotide sequence as set
out herein encompasses a DNA molecule with the specified
sequence, and encompasses a RNA molecule with the specified
sequence in which U is substituted for T, unless context
requires otherwise.

Systems for cloning and expression of a polypeptide in a
variety of different host cells are well known. Suitable host
cells include bacteria, mammalian cells, yeast and baculovirus
systems. Mammalian cell lines available in the art for
expression of a heterologous polypeptide include Chinese
hamster ovary cells, HeLa cells, baby hamster kidney cells,
NSO mouse melanoma cells and many others. A common, preferred
bacterial host is *E. coli*.

The expression of antibodies and antibody fragments in
prokaryotic cells such as *E. coli* is well established in the
art. For a review, see for example Plückthun, A.
Bio/Technology **9**: 545-551 (1991). Expression in eukaryotic
cells in culture is also available to those skilled in the art
as an option for production of a specific binding member, see
for recent reviews, for example Ref, M.E. (1993) Curr. Opinion
Biotech. **4**: 573-576; Trill J.J. et al. (1995) Curr. Opinion
Biotech **6**: 553-560.

Suitable vectors can be chosen or constructed, containing
appropriate regulatory sequences, including promoter
sequences, terminator sequences, polyadenylation sequences,

enhancer sequences, marker genes and other sequences as
appropriate. Vectors may be plasmids, viral e.g. 'phage, or
phagemid, as appropriate. For further details see, for
example, *Molecular Cloning: a Laboratory Manual*: 3nd edition,

5   Sambrook et al., 2001, Cold Spring Harbor Laboratory Press.
Many known techniques and protocols for manipulation of
nucleic acid, for example in preparation of nucleic acid
constructs, mutagenesis, sequencing, introduction of DNA into
cells and gene expression, and analysis of proteins, are

10   described in detail in *Current Protocols in Molecular Biology*,
Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.
The disclosures of Sambrook et al. and Ausubel et al. are
incorporated herein by reference.

15   Thus, a further aspect of the present invention provides a
host cell containing nucleic acid as disclosed herein. A
still further aspect provides a method comprising introducing
such nucleic acid into a host cell. The introduction may
employ any available technique. For eukaryotic cells,

20   suitable techniques may include calcium phosphate
transfection, DEAE-Dextran, electroporation, liposome-mediated
transfection and transduction using retrovirus or other virus,
e.g. vaccinia or, for insect cells, baculovirus. For
bacterial cells, suitable techniques may include calcium

25   chloride transformation, electroporation and transfection
using bacteriophage.

The introduction may be followed by causing or allowing
expression from the nucleic acid, e.g. by culturing host cells

30   under conditions for expression of the gene.

In one embodiment, the nucleic acid of the invention is
integrated into the genome (e.g. chromosome) of the host cell.

Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

5   The present invention also provides a method which comprises using a construct as stated above in an expression system in order to express a specific binding member or polypeptide as above.

10   Further aspects and embodiments of the present invention will be apparent to those skilled in the art in the light of the present disclosure including the following experimental exemplification.

15   All documents mentioned anywhere in this specification and incorporated by reference.

EXPERIMENTAL EXEMPLIFICATION OF ASPECTS AND EMBODIMENTS OF THE PRESENT INVENTION

20

MATERIALS AND METHODS

Preparation and expression of scFv, small immunoprotein (SIP) and IgG1 constructs scFv

25   The scFv(L19) (Figure 1A) is an affinity matured (Kd=5.4x10^{-11}M) antibody fragment specifically directed against the ED-B domain of fibronectin (13 Pini et al., 1998). The scFv(D1.3) (7 McCafferty et al.; 26 Neri et al., 1997), a mouse-anti-hen egg white lysozyme scFv, was used as a control. These scFvs

30   were expressed in *E. Coli* strain HB2151 (Maxim Biotech, San Francisco CA) according to Pini et al. (34 1997).

Mini-immunoglobulin

To construct the L19 small immunoprotein (L19-SIP) gene
(Figure 1C) the DNA sequence coding for the scFv(L19) was
amplified by Polymerase Chain Reaction (PCR) using Pwo DNA

5   Polymerase (Roche), according to manufacturer's
recommendations, with primers BC-618
(gtgtgcactcggaggtgcagctgttggagtctggg - SEQ ID NO. 8) and BC-
619 (gcctccggatttgatttccaccttggtcccttggcc - SEQ ID NO. 9),
containing ApaLI and BspEI restriction sites, respectively.

10 The amplification product was inserted ApaLI/BspEI in the pUT-
εSIP vector, which provides the scFv gene with a secretion
signal, required for secretion of proteins in the
extracellular medium. The pUT-εSIP vector was obtained from
the previously described pUT-SIP-long (33 Li et al., 1997)

15 after substituting the human constant γ1-CH3 domain with the
CH4 domain of the human IgE secretory isoform IgE-S2 (ε_{s2}-CH4;
35 Batista et al., 1996). CH4 is the domain that allows
dimerization in the IgE molecule and the ε_{s2} isoform contains a
cysteine at the carboxyterminal end, which stabilizes the IgE

20 dimer through an inter-chain disulphide bond. In the final SIP
molecule the ScFv(L19) was connected to the ε_{s2}-CH4 domain by a
short GGSG linker. The SIP gene was then excised from the
plasmid pUT-εSIP-L19 with HindIII and EcoRI restriction
enzymes and cloned into the mammalian expression vector pcDNA3

25 (Invitrogen, Groningen, The Netherlands), which contains the
Cytomegalovirus (CMV) promoter, in order to obtain the
construct pcDNA3-L19-SIP.

The DNA sequence coding for scFv(D1.3) was amplified using the

30 primers BC-721 (ctcgtgcactcgcaggtgcagctgcaggagtca - SEQ ID NO.
10) and BC-732 (ctctccggaccgtttgatctcgcgcttggt - SEQ ID NO.
11) and inserted ApaLI/BspEI in the pUT-εSIP vector. The D1.3-

26

SIP gene was then excised from the pUT-εSIP-D1.3 with HindIII
and EcoRI restriction enzymes and cloned into pcDNA3, in order
to obtain the construct pcDNA3-D1.3-SIP.

5   These constructs were used to transfect SP2/0 murine myeloma
cells (ATCC, American Type Culture Collection, Rockville, MD,
USA) using FuGENE 6 Transfection Reagent (Roche), following
the protocol for adherent cells, optimized by the
manufacturer. Transfectomas were grown in DMEM supplemented
10   with 10% FCS and selected using 750 µg/ml of Geneticin (G418,
Calbiochem, San Diego, CA).

IgG1
To prepare complete IgG1, the variable region of the L19 heavy
15   chain (L19-VH), together with its secretion peptide sequence,
was excised with HindIII and XhoI from the previously
described L19-pUTεSIP and inserted in the pUC-IgG1 vector,
containing the complete human γ1 constant heavy chain gene.
The recombinant IgG1 gene was then excised from the pUC-IgG1-
20   L19-VH with HindIII and EcoRI and cloned into pcDNA3, to
obtain the construct pcDNA3-L19-IgG1.

For the preparation of the complete L19 light chain, L19-VL
was amplified from the L19-pUT-εSIP (described above) by PCR
25   using the primers BC-696 (tggtgtgcactcggaaattgtgttgacgcagtc –
SEQ ID NO. 12) and BC-697 (ctctcgtacgtttgatttccaccttggtcc –
SEQ ID NO. 13), containing ApaLI and BsiWI restriction sites,
respectively. After digestion with ApaLI and BsiWI, the
amplification product was inserted in the vector pUT-SEC-hCκ
30   containing the secretion signal sequence and the sequence of
the human constant κ light chain. The recombinant light chain
gene was then excised from pUT-SEC-hCκ-L19-VL with HindIII and
XhoI and inserted in the pCMV2Δ mammalian expression vector,

derived from a pcDNA3 vector by removing the resistance gene
to G418, to obtain the construct pCMV2Δ-L19-κ.

Equimolar amounts of these constructs were used to cotransfect
5   SP2/0 murine myeloma cells as described above. Geneticin
selected clones were screened in ELISA for the ability to
secrete chimeric immunoglobulin, complete of heavy and light
chains.

10 All DNA constructs were purified using the Maxiprep system
from Qiagen (Hilden, Germany), and the DNA sequences of both
strands of the constructs were confirmed using the ABI PRISM
dRhodamine Terminator Cycle Sequencing Ready Reaction Kit
(Perkin Elmer, Foster City, CA). All restriction enzymes (RE)
15 were from Roche Diagnostics (Milan, Italy), with the exception
of BsiWI (New England Biolabs, Beverly, MA). After RE
digestion, inserts and vectors were recovered from agarose
gels using the Qiaquick method (Qiagen).

20 *Purification and quality control of antibodies*
Immunoaffinity chromatography was performed to purify the
different antibodies according to the procedure described by
Carnemolla et al. (24 1996).

25 ED-B conjugated to Sepharose 4B (Amersham Pharmacia Biotech.,
Uppsala, Sweden) following manufacturer's instructions (24
Carnemolla et al., 96) was used to immunopurify all different
L19 antibody formats, while a column of hen egg white lysozyme
(Sigma, St.Louis, USA) conjugated to Sepharose 4B (Amersham
30 Pharmacia) was used for D1.3 antibodies.

The immunopurified antibody formats L19-SIP and L19-IgG1
required no further purification and were dialyzed against

PBS, pH 7.4, at +4°C. Since scFvs obtained from immunoaffinity
chromatography are made up of two forms, monomeric and
dimeric, a second purification step, as described by Demartis
et al. (27 2001), was required to isolate the latter form.

5   Batches of the different antibody formats were prepared and
analyzed using SDS-PAGE under reducing and non-reducing
conditions, immunohistochemistry, size exclusion
chromatography (Superdex 200, Amersham Pharmacia Biotech) and
ELISA experiments.

10

*Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
(SDS-PAGE), Enzyme Linked Immunoabsorbent Assay (ELISA), size
exclusion chromatography and immunohistochemistry*
Screening ELISA experiments on the conditioned culture media

15 were performed according to Carnemolla et al. (24 1996). To
reveal the expression of the different L19 antibody formats,
the recombinant fragment 7B89 (24 Carnemolla et al., 1996),
containing the ED-B domain of FN, that includes the epitope
recognized by the L19, was immobilized on Maxisorp

20 immunoplates (Nunc, Roskilde, Denmark). To detect D1.3
antibodies in ELISA experiments, hen egg white chicken
lysozyme (Sigma) was immobilized on NH2 surface EIA plates
(Costar, Cambridge, MA). A peroxidase-conjugated rabbit anti
human IgE (Pierce, Rockford, IL), diluted according to

25 manufacturer's recommendations, was used as secondary antibody
to detect SIPs. A peroxidase-conjugated rabbit anti human IgG
(Pierce) was used in the case of IgG1. For the scFvs
containing the tag sequence FLAG, a mouse anti-human FLAG
monoclonal antibody (M2, Kodak) and a peroxidase-conjugated

30 goat anti-mouse antibody (Pierce) were used as secondary and
tertiary antibodies, respectively. In all cases the
immunoreactivity with the immobilized antigen was detected

using the substrate ABTS for peroxidase (Roche) and photometric absorbance at 405 nm was measured.

A Superdex 200 (Amersham Pharmacia) chromatography column was used to analyze the gel filtration profiles of the purified antibodies under native conditions using fast protein liquid chromatography (FPLC; Amersham Pharmacia). Immunohistochemistry on different tissue cryostat sections was performed as described by Castellani et al.(22 1994) and 4-18% gradient SDS-PAGE was carried out according to Carnemolla et al. (17 1989) under reducing and non-reducing conditions..

Animals and cell lines
Athymic-nude mice (8 week-old nude/nude CD1 females) were obtained from Harlan Italy (Correzzana, Milano, Italy), 129 (clone SvHsd) strain mice (8-10 weeks old, female) were obtained from Harlan UK (Oxon, England). Mouse embryonal teratocarcinoma cells (F9), human melanoma derived cells (SK-MEL-28) and mouse myeloma cells (SP2/0) were purchased from American Type Culture Collection (Rockville, MD). To induce tumors, nude mice were subcutaneously injected with 16×10^6 SK-MEL-28 cells, and 129 strain mice with 3×10^6 F9 cells. The tumor volume was determined with the following formula: $(d)^2 \times D \times 0,52$, where d and D are, respectively, the short and long dimensions (cm) of the tumor, measured with a caliper. Housing, treatments and sacrifice of animals were carried out according to national legislation (Italian law no. 116 of 27 January, 1992) regarding the protection of animals used for scientific purposes.

Radioiodination of recombinant antibodies
Radioiodination of proteins was achieved following the Chizzonite indirect method (36 Riske et al.,1991) using IODO-

GEN Pre-coated Iodination tubes (Pierce) to activate $Na^{125}I$
(NEN Life Science Products, Boston, MA) according to
manufacturer's recommendations. In the reported experiments,
1.0 mCi of $Na^{125}I$ was used for 0.5mg of protein. The
radiolabeled molecules were separated from free ^{125}I using PD10
(Amersham Pharmacia) columns pre-treated with 0.25% BSA and
equilibrated in PBS. The radioactivity of the samples was
established using a Crystal γ-counter (Packard Instruments,
Milano, Italy). The immunoreactivity assay of the radiolabeled
protein was performed on a 200µl ED-B Sepharose column
saturated with 0.25% BSA in PBS. A known amount of
radioiodinated antibody, in 200γl of 0.25% BSA in PBS, was
applied on top and allowed to enter the column. The column was
then rinsed with 1.5 ml of 0.25% BSA in PBS to remove non-
specifically bound antibodies. Finally, the immunoreactive
bound material was eluted using 1.5 ml of 0.1M TEA, pH11. The
radioactivity of unbound and bound material was counted and
the percentage of immunoreactive antibodies was calculated.
Immunoreactivity was always higher than 90%.

To further analyze the radioiodinated antibodies a known
amount of radiolabeled protein in 200µl was loaded onto the
Superdex 200 column. The retention volume of the different
proteins did not vary after radioiodination. For the three
radioiodinated L19 antibody formats and their negative
controls, the radioactivity recovery from the Superdex 200
column was 100% (Figure 3A, 3B and 3C).

Biodistribution experiments

To block non-specific accumulation of ^{125} Iodine in the stomach
and concentration in thyroid, 30 minutes before injection of
the radiolabeled antibodies mice orally received 20 mg of
sodium perchlorate (Carlo Erba, Italy) in water. This

procedure was repeated at 24h intervals for the duration of biodistribution experiments. Tumor-bearing mice were injected in the tail vein with 0,1 nmoles of the different radiolabeled antibodies (corresponding to 6 µg for scFvs, 8 µg for SIPs and 18 µg for IgGs) in 100µl of saline. Three animals were sacrificed per time point, the different organs including tumor were excised, weighed, counted in a γ-counter and then fixed with 5% formaldehyde in PBS, pH 7.4, to be processed for microautoradiographies, performed according to Tarli et al. (23 1999).

The blood was sampled also for plasma preparation to determine the stability of the radiolabeled molecules in the blood stream using the already described immunoreactivity test and the gel filtration analysis. In both cases 200 µl of plasma were used. The radioactive content of the different organs was expressed as percentage of injected dose per gram (%ID/g). The blood clearance parameters of the radioiodinated antibodies was fitted with a least squares minimization procedure, using the MacIntosh Program Kaleidagraph (Synergy Software, Reading PA, USA) and the equation:

X (t) = A exp (-(alpha t)) + B exp (-(beta t)

where X (t) is the %ID/g of radiolabeled antibody at time t. This equation describes a bi-exponential blood clearance profile, in which the amplitude of the alpha phase is defined as A x 100 / (A + B) and the amplitude of the beta elimination phase is defined as B x 100 / (A + B). Alpha and beta are rate parameters related to the half-lives of the corresponding blood clearance phases. T1/2 (alpha phase) = ln2/alpha = 0.692.../alpha T1/2 (beta phase) = ln2/alpha = 0.692... / alpha. X(0) was assumed to be equal to 40%, corresponding to a blood volume of 2.5 ml in each mouse.

RESULTS

Antibody preparation
Using the variable regions of L19 (13 Pini et al., 1998)
different antibody formats (scFv, mini-immunoglobulin and
complete human IgG1) and their performance _in vivo_ in
targeting tumoral vasculature.

Figure 1 shows the constructs used to express the different
L19 antibody formats. Similar constructs were prepared using
the variable regions of the scFv specific for a non-relevant
antigen (D1.3; 7 McCafferty; 26 Neri et al., 1997).

To obtain SIPs and IgG1, SP2/0 murine myeloma cells were
transfected with the constructs shown in Figure 1 and stable
transfectomas were selected using G418. The best producers
were determined by ELISA and these clones were expanded for
antibody purification. The purification of all three L19
antibody formats was based on immunoaffinity chromatography
using recombinant ED-B conjugated to Sepharose. The yields
were of about 8 mg/l for scFv(L19), 10mg/l for L19-SIP, 3 mg/l
for L19-IgG1. For the control proteins were used scFv(D1.3)
specific for hen-egg lysozyme, and, using the variable regions
of scFv D1.3, D1.3-SIP was constructed. These two antibodies
were purified on hen-egg lysozyme conjugated to Sepharose. The
yields were of 8 and 5 mg/l, respectively. As control for L19-
IgG1 we used commercially available human IgG1/κ (Sigma).

SDS-PAGE analysis of the three purified L19 formats was
performed, under both reducing and non-reducing conditions.
For scFv(L19), the apparent mass was, as expected, about 28
kDa under both reducing and non-reducing conditions (not
shown). The L19-SIP showed a molecular mass of nearly 80 kDa

under non-reducing conditions, and had a mass of about 40 kDa under reducing conditions. The results demonstrated that more than 95% of the native molecule exists as a covalently-linked dimer. L19-IgG1 showed, as expected, a main band of about 180

5   kDa under non-reducing conditions, while, under reducing conditions, it showed two bands corresponding to the heavy chain of about 55 kDa and the light chain of about 28 kDa. Elution profiles of the three L19 antibody formats analyzed by size exclusion chromatography (Superdex 200) were obtained. In

10 all three cases a single peak with a normal distribution, and representing more than 98%, was detected. Using a standard calibration curve, the apparent molecular masses were 60 kDa for scFv(L19)$_2$, 80kDa for L19-SIP and 180kDa for L19-IgG1. In addition, molecular aggregates that are often present in

15 recombinant protein preparations and that may invalidate the results obtained in *in vivo* studies were demonstrated to be absent. SDS-PAGE and size exclusion chromatography (Superdex 200) performed on the purified control proteins gave similar results.

20

Using these three different L19 antibody formats, immunohistochemical analyses were performed on cryostat sections of SK-MEL-28 human melanoma induced in nude mice, and of F9 murine teratocarcinoma induced in 129 strain mice.

25 Optimal results were obtained at concentrations as low as 0.25-0.5 nM. All three purified L19 antibodies recognized identical structures.

In vivo stability of the radiolabeled L19 antibody formats

30 For *in vivo* biodistribution studies, SK-MEL-28 human melanoma and F9 murine teratocarcinoma were used. SK-MEL-28 tumor has a relatively slow growth rate while, F9 tumor grows rapidly (Figure 2). Therefore, the use of SK-MEL-28 tumor enabled

long-lasting experiments (up to 144h), while F9 tumor was
induced for short biodistribution studies (up to 48h). All the
biodistribution experiments were performed when the tumors
were approximately 0.1-0.3 cm^3. For comparison of the various
antibody formats, equimolar amounts (0.1 nmol) in 100µl of
sterile saline were injected. Before injection, the
radioiodinated compounds were filtered 0.22 µm and the
immunoreactivity and gel filtration profile were checked (see
Materials and Methods). Immunoreactivity of the radiolabeled
proteins was always more than 90%.

Figure 3 A-C reports the profiles of the gel filtration
analysis (Superdex 200) of the radioiodinated L19 antibody
formats.

Blood samples were taken from treated animals at the different
time intervals from injection and the radioactivity present in
plasma was analyzed for immunoreactivity and by gel filtration
chromatography. Gel filtration profiles showed a single major
peak, having the molecular mass of the injected protein, for
all three L19 antibody formats. Only the profile of the scFv
revealed a second peak having a higher molecular mass,
suggesting formation of aggregates (Figure 3 D-F).
Furthermore, the formation of large molecular mass aggregates
not eluting from the Superdex 200 column, was observed for
scFv(L19)2. In fact, while the recovery from the Superdex 200
column was 90-100% of the applied radioactivity for both L19-
SIP and L19-IgG, the yield of the loaded radioactivity of
scFv(L19)2 was about 55%. The retained radioactivity was
recovered only after washing the chromatography column with
0.5M NaOH, demonstrating that large aggregates were blocked on
the column filter (Table 1).

Table 1 also reports the results of the immunoreactivity test
performed on plasma (see Materials and Methods). Over the time
of the experiments, L19-SIP and L19-IgG1 maintained the same
immunoreactivity in plasma as the starting reagents. On the
contrary, already 3 hours after injection the immunoreactivity
of scFv(L19)2 in plasma was reduced to less than 40%.

Comparative biodistribution experiments

Tables 2 a, b, c and Figure 4 report the results obtained in
the biodistribution experiments with the radiolabeled L19
antibodies in SK-MEL-28 tumor bearing mice.

Tables 2 a,b,c show, at different times from i.v. injection of
the radiolabeled antibodies, the average (±SD) of the %ID/g of
tissues and organs, including tumors.

In Figure 4 are depicted the variations of the %ID/g of the
different antibody formats in tumor (A) and blood (B) at the
different times of the experiments, as well as the ratios (C)
between the %ID/g in tumor and blood. All three L19 antibody
formats selectively accumulated in the tumor and the ratio of
the %ID/g of tumor and other organs are reported in Table 3.

As demonstrated by microautoradiography, the antibodies
accumulate only on the tumor vasculature, whereas no specific
accumulation on the vasculature of normal organs was seen. By
contrast, no specific accumulation of the radioiodinated
control molecules in either tumors or normal tissues was found
(Tables 2 a, b, c).

All three L19 antibody formats showed a clearance that was
mediated mainly by the kidney, as determined by counting the
urine samples. As expected, clearance rate was faster for

scFv(L19)2 and slower for the complete L19-IgG1. Fitting of
the curve with a biexponential function yielded the half-live
values reported in Table 4.

5   Figure 5 depicts the variations in the %ID/g (±SD) of tumor
and blood obtained with the radioiodinated scFv(L19)2 and L19-
SIP using the F9 teratocarcinoma tumor model. Due to the high
angiogenic activity of F9 teratocarcinoma, accumulation of
radioactive molecules in this tumor was 3 to 4 times higher, 3
10   and 6 h after i.v. injection than in SK-MEL-28 tumor and was
persistently higher for the 48h duration of the experiment. As
for SK-MEL-28 tumor, specific accumulation in tumor
vasculature was confirmed by microautoradiography, while no
specific tumor accumulation was seen after injection of the
15   control molecules. In Table 5 are reported the %ID/g of
L19(scFv) and L19SIP, at different times after i.v. injection,
in F9 tumors and other organs.

Synthesis of reduced L19-SIP
20   To a solution of 375µg (5nmol) L19-SIP in 422µl PBS were added
50µl TCEP-solution (14.34mg TCEPxHCl/5ml aqueous Na_2HPO_4, 0.1M,
pH = 7.4). The reaction mixture was gently shaken for 1h at
37°C. Reduced L19-SIP was purified by gel-chromatography using
a NAP-5 column (Amersham, Eluant: PBS). SDS-PAGE analysis of
25   the isolated product proofed the quantitative transformation
of L19-SIP to reduced L19-SIP.

Yield: 100.3µg/200µl PBS (26.7%).

30   *Synthesis of Tc-99m-L19-SIP*
3.0 mg disodium-L-tartrate were placed in a vial followed by
addition of 100.3µg reduced L19-SIP in 200µl PBS and the
solution was diluted with 100µl aqueous Na_2HPO_4-buffer (1M, pH

= 10.5). 85µl Tc-99m generator eluate (24h) and 10µl SnCl₂-
solution (5mg SnCl₂/1ml 0.1M HCl) were added. The reaction
mixture was shaken for 0.5h at 37°C. Tc-99m-labeled L19-SIP
was purified by gel-chromatography using a NAP-5 column

5   (Amersham, Eluant: PBS).

Radiochemical yield: 35.6%.

Radiochemical purity: 90.2% (SDS-PAGE).

10

Specific activity: 26.4MBq/nmol.

Immunoreactivity: 91.4%

15   *Synthesis of Tc-99m-MAG₂-L19-SIP Carboxy methyl-t-butyl
     disulfide*

A solution of 21.75ml (0.312mol) 1-mercapto-acetic acid,
43.5ml (0.312mol) triethylamine and 100g (0.312mol) N-(tert.-
butylthio)-N,N'-di-BOC-hydrazine in 1l EtOH (abs.) was heated

20   under reflux (N₂-atmosphere) for 60h. EtOH was evaporated under
reduced pressure to a final volume of about 200ml. The residue
was poured in 1.8l H₂O and the pH of the resulting suspension
was adjusted to 7.14 using 5molar NaOH. Di-BOC-hydrazine was
filtered off and the pH of the resulting solution was adjusted

25   to 2.2 using half-concentrated HCl. Crude material was
extracted from water 3x with 600 ml CH₂Cl₂. The combined
organic layers were dried over MgSO₄ and the solvent was
evaporated under reduced pressure yielding 41.1g (80%) as a
yellow oil. The material was pure enough for further

30   synthesis.

N-(benzyloxycarbonyl-Gly)Gly t-butyl ester (Z-(N-Gly)Gly t-butyl ester

A solution of 35.02g (114mmol) Z-Gly-OSuccinimide and 15g (114mmol) Gly-O-^{t}Bu in 1.41 CH_2Cl_2 was stirred under N_2-atmosphere at room temperature for 20 h. The organic layer was washed 3x with 250ml 1% aqueous citric acid, 2x with 200ml half-saturated aqueous NaHCO$_3$ and 1x with 200ml water. The organic layer was dried over anhydrous $MgSO_4$. Evaporation of CH_2Cl_2 under reduced pressure yielded 36.5 g (99%) Z-Gly-Gly-O-^{t}Bu as a yellow oil. The crude material was pure enough for further synthesis.

Gly-Gly t-butyl ester

36.5 g (113 mmol) of Z-Gly-Gly-O^{t}Bu were dissolved in 11 THF followed by the addition of 3.65 g palladium on charcoal (10 %). The mixture was stirred under H_2 atmosphere (1atm) for 3h at room temperature. The suspension was purged with N_2, filtered (PTFE-filter: 0.45µm) and the filtrate was concentrated under reduced pressure yielding 20.3g (95%) Gly-Gly-O-^{t}Bu as a yellow oil. The crude material was pure enough for further synthesis.

Carboxy methyl-t-butyl disulfide glycyl glycine t-butylester

A solution of 23.85g (115.6mmol) DCC in 430ml CH_2Cl_2 was dropwise added to a solution of 21.76 g (115.6 mmol) Gly-Gly-O-^{t}Bu, 20.84 g (115.6 mmol) Carboxy methyl-t-butyl disulfide and 13.3 g (115.6 mmol) NHS in 1 1 CH_2Cl_2. The resulting suspension was stirred over night under N_2-atmosphere at room temperature. After filtration the resulting solution was washed 3x with 400ml half-saturated aqueous NaHCO$_3$ and 1x with 400 ml water. The dried organic layer ($MgSO_4$) was evaporated under reduced pressure. The crude product was purified by chromatography on silica gel using a solvent gradient ranging

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from CH$_2$Cl$_2$/MeOH 99:1 to CH$_2$Cl$_2$/MeOH 98.5:1.5. 26.1g (64 %) were isolated as a yellow oil.

Mercaptoacetyl glycyl glycine

26.32g (75.09mmol) Carboxy methyl-*t*-butyl disulfide glycyl glycine *t*-butyl ester were dissolved in 233ml TFA under N$_2$-atmosphere. The resulting solution was stirred for 20min at room temperature. TFA was evaporated under reduced pressure (5-10 x 10^{-2}mbar) and the resulting oil was dried under stirring for additional 2h (5-10 x 10^{-2}mbar). After addition of 250ml Et$_2$O a white powder precipitated and the suspension was stirred for 3h. The material was filtered off and resuspended in 100ml Et$_2$O. The resulting suspension was stirred over night, the product was filtered off and the material was dried at room temperature under reduced pressure yielding 20.46g (92.5%) as a white powder.

Mercaptoacetyl glycyl glycine NHS ester

Mercaptoacetyl glycyl glycine (1g, 3.4 mmol) and N-hydroxysuccinimide (391 mg, 3.4 mmol) are combined in a dry round bottom flask and dissolved in anhydrous DMF (4 ml). DCC (700 mg, 3.4 mmol) in anhydrous dioxane (2 ml) was added while stirring the reaction mixture. Within 15 min a precipitate (DCU) begins to form. After 1 h the precipitate is removed by vacuum filtration. The precipitate was washed with cold dioxane. The dioxane was removed from the filtrate. The product was precipitated from the remaining DMF solution by adding diethylether. The product was isolated by filtration, washed with cold diethylether, and dried in a vacuum desiccator overnight. Yield: 1.33 (99 %).

Synthesis of Tc-99m-MAG$_2$-ε-HN(Lys)-L19-SIP

200 µg (2.66 nmol) non-reduced L19-SIP in 111 µl PBS were diluted with 300 µl of sodium borate buffer (0.1M, pH 8.5) and

dialyzed 2 x 1 h with 200ml of phosphate buffer (0.1M, pH 8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.). 50 µl of mercaptoacetyl glycyl glycine NHS ester solution (0.50 mg dissolved in 500 µl of phosphate buffer, 0.1M, pH 8.5) were added and the reaction mixture was heated for 3 h at 37°C. The reaction mixture was dialyzed 2 x 1 h and 1 x 17 h (over night) with 200 ml of phosphate buffer (0.1M, pH 8.5) each, employing the Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.). 3.0 mg disodium-L-tartrate were added to the vial followed by addition of. 90 µl Tc-99m generator eluate (eluated daily) and 25µl $SnCl_2$-solution (5mg $SnCl_2$/1ml 0.1M HCl) were added. The reaction mixture was shaken for 0.5h at 37°C. Tc-99m-labeled L19-SIP was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 55.1 %.

Radiochemical purity: 94.5 % (SDS-PAGE).

Specific activity: 15.2 MBq/nmol.

Immunoreactivity: 81.1 %

Synthesis of Re-188-L19-SIP

3.0 mg disodium-L-tartrate were placed in a vial followed by addition of 150µg reduced L19-SIP-SH in 310µl PBS and the solution was diluted with 100µl aqueous Na_2HPO_4-buffer (1M, pH = 10.5). 100µl Re-188 generator eluate and 50µl $SnCl_2$-solution (5mg $SnCl_2$/1ml 0.1M HCl) were added. The reaction mixture was shaken for 1.5h at 37°C. Re-188-labeled L19-SIP was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 34.8 %.

Radiochemical purity: 97.2 % (SDS-PAGE).

Specific activity: 13.5 MBq/nmol.

Immunoreactivity: 91.7 %

5   *Synthesis of reduced L19-SIP for specific conjugation of EDTA,*
       CDTA, TETA, DTPA, TTHA, HBED, DOTA, NOTA, DO3A, and a like
       type chelators to the Cysteine-SH group
       50µl TCEP-solution (14.34mg TCEPxHCl/5ml aqueous Na_2HPO_4, 0.1M,
       pH = 7.4) were added to a solution of 375µg (5 nmol) L19-SIP
10   in 422µl PBS. The reaction mixture was gently shaken for 1h at
       37°C. Reduced L19-SIP was purified by gel-chromatography using
       a NAP-5 column (Amersham, Eluent: sodium acetate buffer, 0.1M,
       pH 5.0). SDS-PAGE analysis of the isolated product proofed the
       quantitative transformation of L19-SIP into reduced L19-SIP.
15   Yield: 105.7µg/200µl (28.2%).

       Synthesis of In-111-MX-DTPA-Maleimide-S(Cys)-L19-SIP-R
       (R = reduced)
20   105 µg (2.8 nmol) reduced L19-SIP in 200 µl of sodium acetate
       buffer (0.1M, pH 5) were reacted with 50µl of dissolved
       1,4,7-triaza-2-(N-maleimido ethylene *p*-amino)benzyl-1,7-
       bis(carboxymethyl)-4-carboxymethyl 6-methyl heptane (0,25mg
       DTPA-Maleimide in 500µl sodium acetate buffer 0.1M pH 5) for
25   3 h at 37°C. The reaction mixture was dialyzed 2 x 1 h with
       200ml of sodium acetate buffer (0.1M, pH 6) employing a Slide-
       A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.).
       80 µl [In-111]$InCl_3$ solution (HCl, 1N, 40 MBq, Amersham Inc.)
       were added and the reaction mixture was heated at 37°C for 30
30   min.

       In-111 labeled DTPA-Maleimide-S(Cys)-L19-SIP was purified by
       gel-chromatography using a NAP-5 column (Amersham, Eluent:
       PBS).

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Radiochemical yield: 51.6 %.

Radiochemical purity: 97.2 % (SDS-PAGE).

Specific activity: 7.9 MBq/nmol.

Immunoreactivity: 88.5 %

Synthesis of MX-DTPA-Maleimide (1,4,7-triaza-2-(N-maleimido
ethylene p-amino)benzyl-1,7-bis(carboxymethyl)-4-carboxymethyl
6-methyl heptane)

512 mg (1 mmol) of {[3-(4-Amino-phenyl)-2-(bis-carboxymethyl-
amino)-propyl]-[2-(bis-carboxymethyl-amino)-propyl]-amino}-
acetic acid (Macrocyclics Inc. Dallas, TX, U.S.A.) and 707 mg
(7 mmol) triethylamine were dissolved in 3 ml dry DMF. 400 mg
(1,5 mmol) of 3-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-propionic
acid 2,5-dioxo-pyrrolidin-1-yl ester (Aldrich) in 1 ml dry DMF
were added drop-wisely. The solution was stirred for 5 h at
50° C. 30 ml of diethylether were added slowly. The reaction
mixture was stirred for further 30 min. The precipitate was
collected by filtering. The crude product was purified by RP-
HPLC (acetonitrile- : water- : trifluoracetic acid / 3 : 96,9
: 0,1 → 99,9 : 0 : 0,1). Yield: 61% (405 mg, 0,61 mmol). MS-
ESI: 664 = M$^+$ +1.

Synthesis of In-111-MX-DTPA-ε-HN(Lys)-L19-SIP

200 µg (2.66 nmol) non-reduced L19-SIP in 111 µl PBS were
diluted with 300 µl of sodium borate buffer (0.1M, pH 8.5) and
dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M, pH
8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc.,
Rockford, IL, U.S.A.). 50 µl of 1,4,7-triaza-2-(*p*-
isothiocyanato)benzyl-1,7-bis(carboxymethyl)-4-carboxymethyl-
6-methyl heptane (MX-DTPA) solution (0.33 mg MX-DTPA dissolved
in 500 µl of sodium borate buffer, 0.1M, pH 8.5) were added
and the reaction mixture was heated for 3 h at 37°C. The

reaction mixture was dialyzed 2 x 1 h and 1 x 17 h (over
night) with 200 ml of sodium acetate buffer (0.1M, pH 6.0)
each, employing the Slide-A-Lyzer 10,000 MWCO (Pierce Inc.,
Rockford, IL, U.S.A.).

80 µl [In-111]InCl$_3$ solution (HCl, 1N, 40 MBq, Amersham Inc.)
were added and the reaction mixture was heated at 37°C for 30
min. In-111 labeled MX-DTPA-ε-HN(Lys)-L19-SIP was purified by
gel-chromatography using a NAP-5 column (Amersham, Eluent:
PBS).

Radiochemical yield: 72.4 %.
Radiochemical purity: 80.3 % (SDS-PAGE).
Specific activity: 8.8 MBq/nmol.
Immunoreactivity: 77.5 %

Synthesis of In-111 -DOTA-C-Benzyl-p-NCS -ε-HN(Lys)-L19-SIP
200 µg (2.66 nmol) non-reduced L19-SIP in 108 µl PBS were
diluted with 300 µl of sodium borate buffer (0.1M, pH 8.5) and
dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M, pH
8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc.,
Rockford, IL, U.S.A.). 50 µl of 1,4,7,10-tetraaza-2-(*p*-
isothiocyanato)benzyl cyclododecane-1,4,7,10-tetraacetic acid
(benzyl-p-SCN-DOTA, Macrocyclics Inc., Dallas TX, U.S.A.)
solution (1.5 mg benzyl-p-SCN-DOTA dissolved in 5 ml of sodium
borate buffer, 0.1M, pH 8.5) were added to the solution and
the reaction mixture was heated for 3 h at 37°C. The reaction
mixture was dialyzed 2 x 1 h and 1 x 17 h (over night) with
200 ml of sodium acetate buffer (0.1M, pH 6.0) each, employing
the Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL,
U.S.A.).

80 μl [In-111]InCl₃ solution (HCl, 1N, 40 MBq, Amersham Inc.) were added and the reaction mixture was heated at 37°C for 30 min. In-111 labeled DOTA-C-Benzyl-p-NCS-ε-HN(Lys)-L19-SIP was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 70.8 %.
Radiochemical purity: 92.1 % (SDS-PAGE).
Specific activity: 10.1 MBq/nmol.
Immunoreactivity: 75.1 %

Synthesis of Y-88-MX-DTPA-ε-HN(Lys)-L19-SIP
200 μg (2.66 nmol) non-reduced L19-SIP in 110 μl PBS were diluted with 300 μl of sodium borate buffer (0.1M, pH 8.5) and dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M, pH 8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.). 50 μl of MX-DTPA solution (0.33 mg MX-DTPA dissolved in 500 μl of sodium borate buffer, 0.1M, pH 8.5) were added and the reaction mixture was heated for 3 h at 37°C. The reaction mixture was dialyzed 2 x 1 h and 1 x 17 h (over night) with 200 ml of sodium acetate buffer (0.1M, pH 6.0) each, employing the Slide-A-Lyzer 10,000 MWCO (Pierce Inc., Rockford, IL, U.S.A.).

100 μl [Y-88]YCl₃ solution (HCl, 1N, 75 MBq, Oak Ridge National Lab.) were added and the reaction mixture was heated at 37°C for 30 min. Y-88 labeled MX-DTPA-ε-HN(Lys)-L19-SIP was purified by gel-chromatography using a NAP-5 column (Amersham, Eluent: PBS).

Radiochemical yield: 68.1 %.
Radiochemical purity: 91.5 % (SDS-PAGE).
Specific activity: 11.4 MBq/nmol.

Immunoreactivity: 70.5%

Synthesis of Lu-177 -DOTA-C-Benzyl-p-NCS -ε-HN(Lys)-L19-SIP

200 µg (2.66 nmol) non-reduced L19-SIP in 120 µl PBS were

dissolved with 300 µl of sodium borate buffer (0.1M, pH 8.5)

and dialyzed 2 x 1 h with 200ml of sodium borate buffer (0.1M,

pH 8.5) employing a Slide-A-Lyzer 10,000 MWCO (Pierce Inc.,

Rockford, IL, U.S.A.). 50 µl of benzyl-p-SCN-DOTA solution

(1.5 mg dissolved in 5 ml of sodium borate buffer, 0.1M, pH

8.5) were added and the reaction mixture was heated for 3 h at

37°C. The reaction mixture was dialyzed 2 x 1 h and 1 x 17 h

(over night) with 200 ml of sodium acetate buffer (0.1M, pH

6.0) each, employing the Slide-A-Lyzer 10,000 MWCO (Pierce

Inc., Rockford, IL, U.S.A.).

200 µl [Lu-177]$LuCl_3$ solution (HCl, 1N, 80 MBq, NRH-Petten,

Netherlands) were added and the reaction mixture was heated at

37°C for 30 min. Lu-177 labeled DOTA-C-Benzyl-p-NCS-ε-HN(Lys)-

L19-SIP was purified by gel-chromatography using a NAP-5

column (Amersham, Eluent: PBS).

Radiochemical yield: 72.2 %.
Radiochemical purity: 94.9 % (SDS-PAGE).
Specific activity: 18.3 MBq/nmol.
Immunoreactivity: 73.4 %

Organ distribution and excretion of In-111-MX-DTPA-L19-SIP
after a single i.v. injection into tumour-bearing nude mice

The labeled peptides of the invention were injected

intravenously in a dose of about 37 kBq into F9

(teratocarcinoma)-bearing animals (body weight about 25 g).

The radioactivity concentration in various organs, and the

radioactivity in the excreta, was measured using a γ counter
at various times after administration of the substance.

The biodistribution of In-111-MX-DTPA-L19-SIP in F9
(teratocarcinoma)-bearing nude mice (mean ± SD, n=3) is shown
in Table 6.

*Organ distribution and excretion of Tc-99m-L19-SIP after a
single i.v. injection into tumour-bearing nude mice*

Labeled peptides were injected intravenously in a dose of about
56 kBq into F9 (teratocarcinoma)-bearing animals (bodyweight
about 25 g). The radioactivity concentration in various organs,
and the radioactivity in the excreta was measured using a γ
counter at various times after administration of the substance.
In addition, the tumour to blood ratio was found at various
times on the basis of the concentration of the peptide in tumour
and blood.

The biodistribution of Tc-99m-L19-SIP in F9 (teratocarcinoma)-
bearing nude mice (mean ± SD, n=3) is shown in Table 7.

The tumour to blood ratio of Tc-99m-L19-SIP in F9
(teratocarcinoma)-bearing nude mice (mean ± SD, n=3) is shown
in Table 8.

Radiolabeled peptides proved to possess favorable properties in
animal experiments. For example, Tc-99m-L19-SIP and In-111-MX-
DTPA-ε-HN(Lys)-L19-SIP displayed high tumor accumulation of
17.2 (Tc-99m) or 12.9 (In-111) % injected dose per gram (ID/g)
at 1 hour post injection (p.i.). Significant tumor retention of
9.4 (Tc-99m) or 13.0 (In-111) % ID/g at 24 hours p.i. was
observed. Thus, tumor uptake is significantly higher compared to

other known In-111 or Tc-99m labeled antibody fragments (e.g.
Kobayashi et al., J. Nuc. Med., Vol. 41(4), pp. 755 - 762, 2000;
Verhaar et al., J. Nuc. Med., Vol. 37(5), pp. 868 - 872, 1996).
The compound's blood clearance lead to tumor/blood ratios of
13:1 and 6:1 respectively, at 24 h p.i.

Most remarkably In-111-MX-DTPA-ε-HN(Lys)-L19-SIP displayed
significantly lower kidney uptake and retention (22.5 % ID/g)
than other highly retained In-111 labeled recombinant antibody
fragment (120 % ID/g) described e.g. by Kobayashi et al. at 24 h
p.i. Kidney retention is a very common problem and usually
hampers the use of lanthanide labeled compounds in radiotherapy.

The experimental results demonstrate the excellent potential
of the radioimmunoconjugates described herein for diagnostic
and therapeutic applications, preferably applied to the
patient by parenteral administration.

DISCUSSION
The observation that cytotoxic anticancer drugs localize more
efficiently in normal tissues than in tumors (37 Bosslet et
al., 1998) prompted a wave of studies investigating the
possibility of selective drug delivery to tumors. The
effective targeting of tumors, however, has two main
requisites: 1) a target in the tumor that is specific,
abundant, stable and readily available for ligand molecules
coming from the bloodstream, and 2) a ligand molecule with
suitable pharmakokinetic properties that is easily diffusible
from the bloodstream to the tumor and with a high affinity for
the target to ensure its efficient and selective accumulation
in the tumor.

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Due to its distinctive features the tumor microenvironment is a possible pan-tumoral target. In fact, tumor progression induces (and subsequently needs) significant modifications in tumor micro-environment components, particularly those of the extracellular matrix (ECM). The molecules making up the ECM of solid tumors differ both quantitatively and qualitatively from those of the normal ECM. Moreover, many of these tumor ECM components are shared by all solid tumors, accounting for general properties and functions such as cell invasion (both normal cells into tumor tissues and cancer cells into normal tissues) and angiogenesis. Of the numerous molecules constituting the modified tumor ECM, the present inventors have focused attention on a FN isoform containing the ED-B domain (B-FN).

B-FN is widely expressed in the ECM of all solid tumors thus far tested and is constantly associated with angiogenic processes (22 Castellani et al., 1994), but is otherwise undetectable in normal adult tissues (17 Carnemolla et al., 1989). Targeted delivery of therapeutic agents to the subendothelial ECM overcomes problems associated with interstitial hypertension of solid tumors (38 Jain et al. 1988; 39 Jain, 1997; 40 Jain RK, 1999).

L19 (13 Pini et al. 1998; 25 Viti, Canc.Res., 23 Tarli, et al., 1999), an scFv with a high affinity (Kd=5.4×10^{-11}M) for the ED-B domain of FN, selectively and efficiently accumulates *in vivo* around tumor neo-vasculature and is able to selectively transport and concentrate in the tumor mass any one of a number of therapeutic molecules to which it is conjugated (28 Birchler et al., 1999; 29 Nilsson, et al., 2001; 30 Halin et al.2002; 31 Carnemolla et al., 2002). The

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ability of L19 to selectively target tumors has also been
demonstrated in patients using scintigraphic techniques.

5 The present specification reports on tumor vascular targeting
performance and pharmacokinetics of three different L19 human
antibody formats: the scFv, the mini-immunoglobulin/small
immunoprotein (SIP) and complete human IgG1.

The SIP molecule was obtained by fusion of the scFv(L19) to
10 the εCH4 domain of the secretory isoform S_2 of human IgE. The
εCH4 is the domain that allows dimerization of IgE molecules
and the S_2 isoform contains a cysteine at the COOH terminal
that covalently stabilizes the dimer through an interchain
disulphide bond (35 Batista et al., 1996). The IgE binding
15 sites for FcεRI reside in the CH3 domain (41 Turner and Kinet,
1999; 42 Vangelista et al., 1999; 43 Garman et al., 2000), so
scFv fused to εCH4 domain in accordance with embodiments of
the present invention does not activate any signalling leading
to hypersensitivity reactions.
20
The performance of these three formats in two different tumor
models in mouse has been studied: in murine F9 teratocarcinoma
and human SK-MEL-28 melanoma. The first is a rapidly growing
tumor that, once implanted, kills the animals in about two
25 weeks. SK-MEL-28 tumor, on the other hand, presents a biphasic
growth curve, with an early, fast, growth phase followed by a
second, slower, phase. It has previously been shown that the
amount of ED-B in F9 teratocarcinoma remains stable during
tumor growth (23 Tarli, et al., 1999); by contrast, ED-B
30 accumulates in SK-MEL-28 melanoma proportionally to the
ability of the tumor to grow (23 Tarli et al., 1999), with
abundant ED-B being found in the first phase and a lesser
amount in the second. The use of SK-MEL-28 melanoma tumor

allowed long-term biodistribution studies without dramatic
variations of tumoral mass (Figure 2) that could give rise to
misinterpretation of results.

5   Comparative studies of the three L19 antibody formats in terms
of stability *in vivo* showed that L19-SIP and L19-IgG1
maintained, for the duration of experiments (144h), the same
immunoreactivity and molecular mass in plasma as before
injection. By contrast, scFv(L19) rapidly lost its

10   immunoreactivity in plasma and generated aggregates that were
too large to enter the gel filtration chromatography column.
Such aggregation of the scFv is very likely responsible for
the ratio between %ID/g of tumor and lung, since aggregates
could accumulate in the microvasculature of the lung (Table

15   3). For all three formats, the blood clearance is mediated
mainly via the kidney, showing a biphasic curve with an α and
a β phase, reported in Table 4, which is inversely
proportional to molecular size.

20   The accumulation of the different antibody formats in the
tumors studied was a consequence of the clearance rate and *in
vivo* stability of the molecules. Using the scFv, the maximum
percent injected dose per gram (%ID/g) was observed 3h after
injection of the radiolabeled antibody and then rapidly

25   decreased. Using the SIP, the %ID/g in tumors was 2-5 times
higher than that of the scFv, reaching a maximum 4-6 hours
after injection. This pattern was observed in both F9 and SK-
MEL-28 tumors. By contrast, the accumulation of IgG1 in tumors
rose constantly during the experiments. However, due to its

30   slow clearance, the tumor-blood ratio of the %ID/g after 144
hours was only about 3, compared to a ratio of 10 for the scFv
and 70 for the SIP after the same period of time (Figure 4).

The same distinctive properties of *in vivo* stability,
clearance and tumor targeting performance shown by the three
antibody formats studied here may be exploited for different
diagnostic and/or therapeutic purposes, depending on the
5   clinical needs and disease. For instance, radiolabeled
antibodies showing good tumor-organ and tumor-blood ratios
soon after injection are necessary for *in vivo* diagnostic
immunoscintigraphy, mainly because short half-life isotopes
are used in such analysis.

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Different approaches are possible using antibody as a vehicle
for therapeutic agents: delivery of substances that display
their therapeutic effects after reaching their targets (e.g.,
photosensitisers activated only on the targets), for which the
15   absolute amount delivered to the tumor is relevant; delivery
of substances that exert their therapeutic and toxic effects
even before reaching the target (e.g., the β-emitter Yttrium-
90), for which particular attention must be given to the ratio
of the area under the curves of tumor and blood accumulation
20   as a function of time, in order to minimize the systemic
toxicity and to maximize the anti-tumor therapeutic effect.

L19-SIP, for instance, seems to offer the best compromise of
molecular stability, clearance rate and tumor accumulation.
25   Similar fusion proteins composed of scFv antibody fragments
bound to a dimerizing domain have already been described (44
Hu et al, 1996; 33 Li et al., 1997), but in both cases the
human γ1CH3 was used as the dimerizing domain. The usage of
the human ε_{s2}CH4 domain provides an easy way of getting a
30   covalent stabilization of the dimer. In addition, the
disulphide bridge formed by the C-terminal cysteine residues
can be easily reduced in mild enough conditions to preserve
the overall structure of the molecule, thus providing a

readily accessible reactive group for radiolabelling or chemical conjugation. This feature seems particularly promising in the view of the clinical potential.

L19-IgG1 gathers abundantly in tumors, and even though this accumulation is offset by a slow blood clearance rate, the three step procedure to remove circulating antibodies may be used to allow its use not only for therapeutic purposes but also for diagnostic immunoscintigraphy (45 Magnani et al. 2000).

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Table 1. Immunoreactivity (I*) and radioactivity recovery (R) from Superdex 200 of the radiolabeled antibodies, at different times after i.v. injection

Time (h)	3		6		24		48		72		144	
	I	R	I	R	I	R	I	R	I	R	I	R
L19(scFv)	36	54	32	58	27	nd	14	nd	9	nd	4	nd
L19SIP	100	100	100	96	100	94	95	96	100	nd	95	nd
L19IgG1	100	100	100	100	95	100	100	100	100	100	95	100

Immunoreactivity (%) and radioactivity recovery (%) from Superdex200 were determined in plasma as described in Materials and Methods.

- *To normalize, the results of the immunoreactivity test are referred to the percentage values of the immunoreactivity before i.v. injection.

- nd : not determined

Table 2 a. Biodistribution experiments of radiolabeled L19 and D1.3 antibody fragments in SK-MEL-28 tumor-bearing mice

L19(scFv)

	3h	6h	24h	48h	72h	144h
TUMOR	2,47 ± 0,65	2,01 ± 0,72	1,62 ± 0,43	0,95 ± 0,14	0,68 ± 0,04	0,32 ±0,14
Blood	1,45 ± 0,58	0,54 ± 0,12	0,10 ± 0,03	0,04 ± 0,01	0,03 ± 0,02	0,03 ±0,01
Liver	0,48 ± 0,20	0,18 ± 0,05	0,04 ± 0,01	0,02 ± 0,00	0,02 ± 0,01	0,02 ±0,00
Spleen	0,67 ± 0,28	0,27 ± 0,04	0,07 ± 0,02	0,03 ± 0,00	0,02 ± 0,01	0,02 ±0,00
Kidney	4,36 ± 0,32	1,67 ± 0,08	0,16 ± 0,01	0,06 ± 0,01	0,04 ± 0,02	0,03 ±0,00
Intestine	0,77 ± 0,21	0,57 ± 0,05	0,24 ± 0,06	0,17 ± 0,04	0,12 ± 0,05	0,09 ±0,01
Heart	0,77 ± 0,20	0,31 ± 0,07	0,07 ± 0,02	0,02 ± 0,00	0,02 ± 0,01	0,02 ±0,00
Lung	2,86 ± 0,34	1,50 ± 0,67	1,07 ± 0,42	0,73 ± 0,39	0,55 ± 0,11	0,51 ±0,22

D1.3(scFv)

	3h	6h	24h	48h	72h	144h
TUMOR	1,03 ± 0,74	0,87 ± 0,42	0,15 ± 0,10	0,07 ± 0,02	nd	nd
Blood	1,52 ± 0,86	0,81 ± 0,13	0,02 ± 0,00	0,01 ± 0,00	nd	nd
Liver	1,19 ± 0,65	0,66 ± 0,26	0,14 ± 0,04	0,03 ± 0,08	nd	nd
Spleen	1,05 ± 0,88	0,42 ± 0,33	0,07 ± 0,02	0,05 ± 0,01	nd	nd
Kidney	3,01 ± 2,48	1,83 ± 0,76	0,48 ± 0,01	0,18 ± 0,05	nd	nd
Intestine	0,56 ± 0,54	0,56 ± 0,13	0,17 ± 0,03	0,02 ± 0,01	nd	nd
Heart	0,86 ± 0,54	0,55 ± 0,84	0,02 ± 0,01	0,01 ± 0,00	nd	nd
Lung	1,28 ± 0,65	1,06 ± 0,88	0,04 ± 0,01	0,03 ± 0,01	nd	nd

The results are expressed as percent of antibody injected dose per gram of tissue (%ID/g)±SD
nd: not determined

Table 2 b. Biodistribution experiments of radiolabeled L19-SIP and D1.3-SIP in SK-MEL-28 tumor-bearing mice

L19 SIP

	3h	6h	24h	48h	72h	144h
TUMOR	5,23 ± 0,65	6,14 ± 2,23	4,20 ± 2,47	2,57 ± 0,31	2,33 ± 0,90	1,49 ± 0,65
Blood	9,82 ± 0,68	5,03 ± 0,52	1,39 ± 0,06	0,29 ± 0,04	0,08 ± 0,02	0,02 ± 0,01
Liver	2,65 ± 0,14	1,74 ± 0,31	0,50 ± 0,04	0,19 ± 0,01	0,10 ± 0,02	0,05 ± 0,01
Spleen	3,76 ± 0,36	2,43 ± 0,24	0,71 ± 0,05	0,26 ± 0,04	0,13 ± 0,01	0,17 ± 0,18
Kidney	7,33 ± 0,91	3,87 ± 0,21	1,09 ± 0,05	0,30 ± 0,04	0,14 ± 0,02	0,05 ± 0,01
Intestine	1,45 ± 0,24	1,44 ± 0,29	1,06 ± 0,43	0,56 ± 0,08	0,40 ± 0,08	0,18 ± 0,00
Heart	4,16 ± 0,30	2,15 ± 0,08	0,52 ± 0,05	0,13 ± 0,03	0,06 ± 0,01	0,02 ± 0,01
Lung	7,72 ± 0,60	5,41 ± 0,55	1,81 ± 0,40	0,59 ± 0,29	0,19 ± 0,03	0,05 ± 0,01

D1.3SIP

	3h	6h	24h	48h	72h	144h
TUMOR	3,80 ± 0,30	1,65 ± 0,12	0,70 ± 0,00	0,26 ± 0,01	0,07 ± 0,01	0,04 ± 0,03
Blood	10,40 ± 0,81	4,45 ± 0,14	1,21 ± 0,01	0,32 ± 0,00	0,08 ± 0,01	0,06 ± 0,02
Liver	4,05 ± 0,98	2,73 ± 0,33	1,43 ± 0,07	0,51 ± 0,21	0,15 ± 0,08	0,02 ± 0,01
Spleen	3,31 ± 0,66	1,76 ± 0,50	0,82 ± 0,12	0,46 ± 0,20	0,15 ± 0,05	0,04 ± 0,02
Kidney	8,41 ± 0,49	4,64 ± 0,06	1,47 ± 0,05	0,36 ± 0,03	0,16 ± 0,03	0,06 ± 0,01
Intestine	2,03 ± 0,55	1,06 ± 0,20	1,02 ± 0,06	0,14 ± 0,03	0,08 ± 0,02	0,12 ± 0,04
Heart	3,28 ± 0,20	1,81 ± 0,02	0,29 ± 0,01	0,06 ± 0,00	0,05 ± 0,01	0,04 ± 0,01
Lung	6,16 ± 0,28	4,52 ± 0,07	1,16 ± 0,05	0,09 ± 0,00	0,06 ± 0,01	0,05 ± 0,01

The results are expressed as percent of antibody injected dose per gram of tissue (%ID/g)±SD

nd: not determined

Table 2 c. Biodistribution experiments of radiolabeled L19IgG1 and hIgG1k in SK-MEL-28 tumor-bearing mice

L19 IgG1

	3h	6h	24h	48h	72h	144h
TUMOR	4,46 ± 0,08	5,39 ± 1,01	6,70 ± 2,10	7,80 ± 2,51	8,90 ± 2,52	11,22 ± 3,19
Blood	16,04 ± 0,81	12,02 ± 1,65	8,31 ± 1,77	5,12 ± 1,42	5,02 ± 3,81	4,87 ± 0,26
Liver	6,03 ± 0,37	6,77 ± 0,53	2,41 ± 0,35	1,45 ± 0,41	1,26 ± 0,71	1,09 ± 0,16
Spleen	6,63 ± 1,34	6,37 ± 1,37	2,51 ± 0,47	2,01 ± 0,32	1,90 ± 1,02	1,51 ± 0,29
Kidney	6,47 ± 0,39	5,12 ± 0,47	3,07 ± 0,35	1,73 ± 0,63	1,54 ± 1,14	1,12 ± 0,44
Intestine	1,60 ± 0,39	1,35 ± 0,65	1,43 ± 0,19	1,13 ± 0,32	1,13 ± 0,98	0,97 ± 0,47
Heart	5,63 ± 0,67	4,77 ± 0,52	2,87 ± 0,45	1,48 ± 0,51	1,32 ± 1,09	0,92 ± 0,37
Lung	6,55 ± 0,65	5,15 ± 0,62	4,16 ± 0,66	2,28 ± 0,80	1,98 ± 1,60	1,42 ± 0,45

hIgG1k

	3h	6h	24h	48h	72h	144h
TUMOR	nd	3,28 ± 0,38	4,00 ± 0,22	2,78 ± 0,20	nd	2,32 ± 0,26
Blood	nd	10,12 ± 0,35	7,87 ± 0,25	6,24 ± 0,34	nd	5,41 ± 0,51
Liver	nd	4,02 ± 0,09	2,06 ± 0,10	1,90 ± 0,24	nd	1,28 ± 0,03
Spleen	nd	4,47 ± 0,28	1,82 ± 0,01	1,42 ± 0,19	nd	1,24 ± 0,03
Kidney	nd	5,40 ± 0,19	2,56 ± 0,06	2,08 ± 0,22	nd	1,30 ± 0,15
Intestine	nd	0,72 ± 0,07	0,46 ± 0,05	0,36 ± 0,03	nd	0,31 ± 0,01
Heart	nd	3,80 ± 0,15	2,52 ± 0,21	0,99 ± 0,18	nd	1,48 ± 0,13
Lung	nd	4,82 ± 0,92	3,64 ± 0,08	1,75 ± 0,32	nd	1,09 ± 0,13

The results are expressed as percent of antibody injected dose per gram of tissue (%ID/g)±SD

nd: not determined

Table 3. Tumor-organ ratios of the % ID/g of the radiolabeled L19 antibody formats in SK-MEL-28 tumor-bearing mice.

Time(h)	L19(ScFv)						L19SIP						L19IgG1					
	3	6	24	48	72	144	3	6	24	48	72	144	3	6	24	48	72	144
TUMOR	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Blood	1,7	3,7	16,2	23,7	22,7	10,7	0,5	1,2	3,0	8,9	29,1	74,5	0,3	0,4	0,8	1,5	1,8	2,3
Liver	5,1	11,1	40,5	47,5	34,0	16,0	2,0	3,5	8,4	13,5	23,3	29,8	0,7	0,8	2,8	5,4	7,1	6,3
Spleen	3,7	7,4	23,1	31,6	34,0	16,0	1,4	2,5	5,9	10,0	17,9	8,8	0,7	0,6	2,7	3,9	4,9	7,4
Kidney	0,6	1,2	10,1	15,8	17,0	10,7	0,7	1,6	3,8	8,6	16,6	29,8	0,7	1,0	2,2	4,5	5,8	5,3
Intestine	3,2	3,5	6,7	5,6	5,7	3,6	3,6	4,3	4,0	4,6	5,8	8,3	2,8	4,0	4,7	6,9	7,9	7,1
Heart	3,2	6,5	23,1	47,5	34,0	16,0	1,3	2,9	8,1	20,0	38,8	74,5	0,8	1,1	2,3	5,3	6,7	5,8
Lung	0,9	1,3	1,5	1,3	1,2	0,6	0,7	1,1	2,3	4,3	12,3	29,8	0,7	1,0	1,6	3,4	4,5	3,7

Table 4.

Kinetic parameters for blood clearance of the three L19 antibody formats

	α		β	
	(%)[a]	$t_{1/2}$ (h)	(%)[a]	$t_{1/2}$ (h)
L19(scFv)	96,7	0,53	3,3	8,00
L19-SIP	83,7	1,06	16,3	10,66
L19-IgG1	76,9	1,48	23,1	106,7

[a] Relative magnitude of the two half-life components

Table 5.Biodistribution experiments of radiolabeled L19(scFv) and L19SIP in F9 tumor-bearing mice

L19(scFv)	3h	6h	24h	48h
TUMOR	10,46 ±1,75	8,15 ±2,63	3,18 ±0,83	2,83 ±0,71
Blood	2,05 ±0,38	1,88 ±1,14	0,17 ±0,01	0,06 ±0,02
Liver	1,62 ±1,67	0,73 ±0,51	0,07 ±0,01	0,04 ±0,02
Spleen	1,53 ±0,36	0,90 ±0,54	0,11 ±0,01	0,05 ±0,01
Kidney	12,70 ±0,73	4,37 ±0,98	0,24 ±0,03	0,18 ±0,08
Intestine	0,68 ±0,15	0,95 ±0,23	0,24 ±0,01	0,17 ±0,06
Heart	1,35 ±0,21	0,81 ±0,38	0,08 ±0,02	0,04 ±0,01
Lung	2,88 ±0,29	2,06 ±0,69	0,38 ±0,60	0,33 ±0,05

L19SIP	3h	6h	24h	48h
TUMOR	17,46 ±1,93	16,65 ±2,59	15,32 ±2,17	12,00 ±1,91
Blood	13,51 ±0,57	9,62 ±1,18	1,73 ±0,02	1,14 ±0,20
Liver	2,81 ±0,37	2,39 ±0,13	0,54 ±0,14	0,32 ±0,00
Spleen	3,42 ±0,26	2,66 ±0,27	0,61 ±0,09	0,37 ±0,01
Kidney	9,18 ±0,76	5,85 ±0,50	1,16 ±0,05	0,76 ±0,06
Intestine	0,95 ±0,03	1,36 ±0,21	0,83 ±0,11	1,04 ±0,14
Heart	4,64 ±0,24	3,67 ±0,46	0,67 ±0,06	0,46 ±0,07
Lung	5,61 ±0,01	5,93 ±0,57	1,66 ±0,19	0,91 ±0,08

The results are expressed as percent of antibody injected dose per gram of tissue (%ID/g)±SD

nd: not determined

	% of dose / g of tissue		
	1h p.i.	3h p.i.	24h p.i.
Spleen	5.05 ± 1.04	4.27 ± 0.27	4.86 ± 1.77
Liver	10.80 ± 1.52	10.57 ± 1.44	10.68 ± 1.51
Kidney	14.30 ± 1.45	16.71 ± 2.42	22.48 ± 6.79
Lung	9.94 ± 1.72	6.15 ± 0.80	3.03 ± 0.95
Stomach without contents	1.10 ± 0.13	1.62 ± 0.19	1.66 ± 0.24
Intestine with contents	1.67 ± 0.14	2.65 ± 0.30	2.64 ± 1.40
Tumour	12.93 ± 2.76	10.18 ± 2.28	12.96 ± 3.13
Blood	17.10 ± 1.49	9.08 ± 0.96	1.98 ± 0.47

Table 6

	% of dose / g of tissue					
	1h p.i.		3h p.i.		24h p.i.	
Spleen	6.92 ±	1.3	5.37 ±	0.23	2.06 ±	0.48
Liver	14.65 ±	0.81	12.43 ±	0.37	4.62 ±	0.52
Kidney	22.07 ±	1.87	15.99 ±	1.10	5.92 ±	1.18
Lung	10.06 ±	1.67	5.33 ±	0.49	1.32 ±	0.25
Stomach without contents	2.18 ±	0.39	2.12 ±	0.09	1.15 ±	0.08
Intestine with contents	3.03 ±	0.25	3.62 ±	0.58	1.20 ±	0.12
Tumour	17.20 ±	7.49	18.79 ±	5.35	9.42 ±	3.84
Blood	16.53 ±	2.04	7.42 ±	0.21	0.73 ±	0.14

Table 7

	1h p.i.	3h p.i.	24h p.i.
Tumour to blood ratio	1.01 ± 0.33	2.54 ± 0.74	12.81 ± 4.03

Table 8

CLAIMS:

1.   A specific binding member that binds human ED-B and which
comprises an antigen-binding site that comprises an antibody
5   VH domain and an antibody VL domain, wherein the antibody VH
domain is selected from the group consisting of the L19 VH
domain, and a VH domain comprising a VH CDR1, a VH CDR2 and a
VH CDR3, wherein the VH CDR3 is the L19 VH CDR3 of SEQ ID NO.
3, the VH CDR1 is optionally L19 VH CDR1 of SEQ ID NO. 1, and
10   the VH CDR2 is optionally L19 VH CDR2 of SEQ ID NO. 2; and
wherein the antibody VL domain is optionally selected from the
group consisting of the L19 VL domain, and a VL domain
comprising a VL CDR1, a VL CDR2 and a VL CDR3, wherein the VL
CDR3 is the L19 VL CDR3 of SEQ ID NO. 6, the VL CDR1 is
15   optionally L19 VL CDR1 of SEQ ID NO. 4, and the VL CDR2 is
optionally L19 VL CDR2 of SEQ ID NO. 5; the L19 VH domain and
L19 VL domain sequences being disclosed in Pini et al. (1998)
J. Biol. Chem. 273: 21769-21776; wherein the specific binding
member comprises a mini-immunoglobulin comprising said
20   antibody VH domain and antibody VL domain fused to ε_{s2}-CH4 and
dimerized or comprises a whole IgG1 antibody molecule.

2.   A specific binding member according to claim 1 comprising
an antibody VH domain comprising the VH CDR's with the amino
25   acid sequences of SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO. 3,
which specific binding member competes for binding to ED-B
with an ED-B-binding domain of an antibody comprising the L19
VH domain and the L19 VL domain.

30   3.   A specific binding member according to claim 1 or claim 2
comprising the L19 VH domain.

4.   A specific binding member according to claim 3 comprising the L19 VL domain.

5.   A specific binding member according to any one of the preceding claims which is a mini-immunoglobulin comprising ε_{s2}-CH4.

6.   A specific binding member according to claim 5 wherein the antibody VH domain and antibody VL domain are within an scFv antibody molecule fused to ε_{s2}-CH4.

7.   A specific binding member according to claim 6 wherein the scFv antibody molecule is fused to ε_{s2}-CH4 via a linker peptide.

8.   A specific binding member according to claim 7 wherein the linker peptide has the amino acid sequence GGSG (SEQ ID NO. 7).

9.   A specific binding member according to any one of claims 1 to 4 that comprises a whole IgG1 antibody molecule.

10.   A specific binding member according to any of claims 1 to 9 which is conjugated to a radioisotope

11.   A specific binding member according to claim 10 wherein the radioisotope is a radioisotope of Tc, Re, In, Y or Lu.

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12.   A specific binding member according to claim 10 wherein the radioisotope is selected from the group consisting of ^{94m}Tc, ^{99m}Tc, ^{186}Re, ^{203}Pb, ^{67}Ga, ^{68}Ga, ^{43}Sc, ^{47}Sc, ^{110m}In, ^{111}In, ^{97}Ru, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{68}Cu, ^{86}Y, ^{88}Y, ^{90}Y, ^{121}Sn, ^{161}Tb, ^{153}Sm, ^{166}Ho, ^{105}Rh, ^{177}Lu, ^{172}Lu and ^{18}F.

13.   An isolated nucleic acid which comprises a nucleotide sequence or nucleotide sequences encoding a specific binding member according to any one of claims 1 to 9.

14.   A host cell transformed with nucleic acid according to claim 13.

15.   A method of producing a specific binding member, the method comprising culturing host cells according to claim 14 under conditions for production of said specific binding member.

16.   A method according to claim 15 further comprising isolating and/or purifying said specific binding member.

17.   A method according to claim 15 or claim 16 further comprising formulating the specific binding member into a composition including at least one additional component.

18.   A method according to any one of claims 15 to 17 further comprising binding the specific binding member to ED-B or a fragment of ED-B.

19.   A method comprising binding a specific binding member that binds ED-B according to any one of claims 1 to 9 to ED-B or a fragment of ED-B.

20.   A method according to claim 18 or claim 19 wherein said
binding takes place *in vitro*.

21.   A method according to any one of claims 18 to 20
comprising determining the amount of binding of specific
binding member to ED-B or a fragment of ED-B.

22.   A composition comprising a specific binding member
according to any one of claims 1 to 9, for use in a method of
treatment of the human or animal body by therapy.

23.   A composition according to claim 22 for use in a method
of treatment of a lesion of pathological angionesis.

24.   A composition according to claim 22 for use in a method
of treatment of a tumor.

25.   Use of a specific binding member according to any one of
claims 1 to 9 in the manufacture of a medicament for treating
a lesion of pathological angiogenesis.

26.   Use of a specific binding member according to any one of
claims 1 to 9 in the manufacture of a medicament for treating
a tumor.

**Figure 1A**
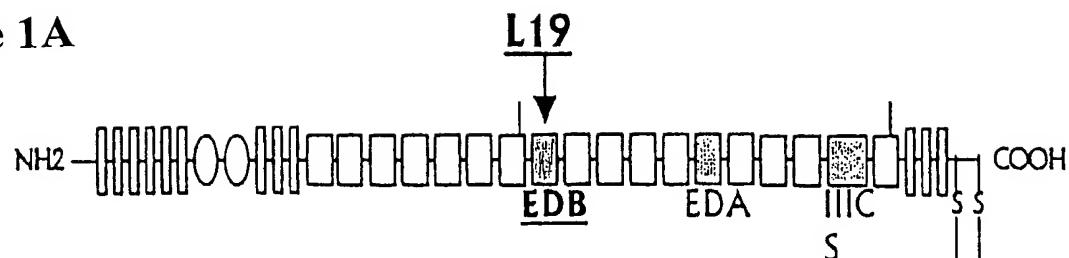
Figure 1B

L19scFv/pDN322
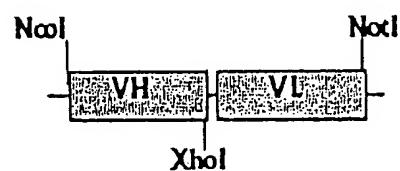
Figure 1C

L19SIP/pCDNA3
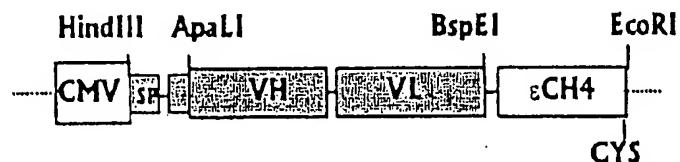
Figure 1D

L19HIgG1/pcDNA3
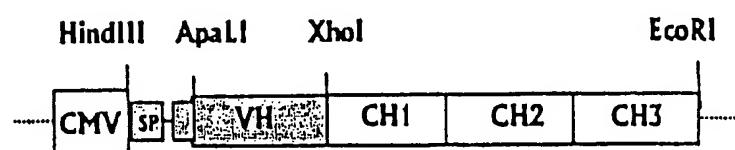
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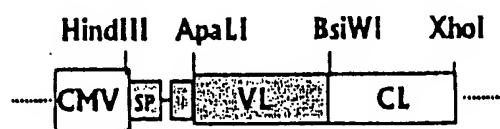
Figure 2

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Figure 3A

^{125}I-L19 (scFv)

Figure 3D

^{125}I-L19 (scFv)

Figure 3B

^{125}I-L19 SIP

Figure 3E

^{125}I-L19 SIP

Figure 3C

^{125}I-L19 IgG1

Figure 3F ^{125}I-L19 IgG1
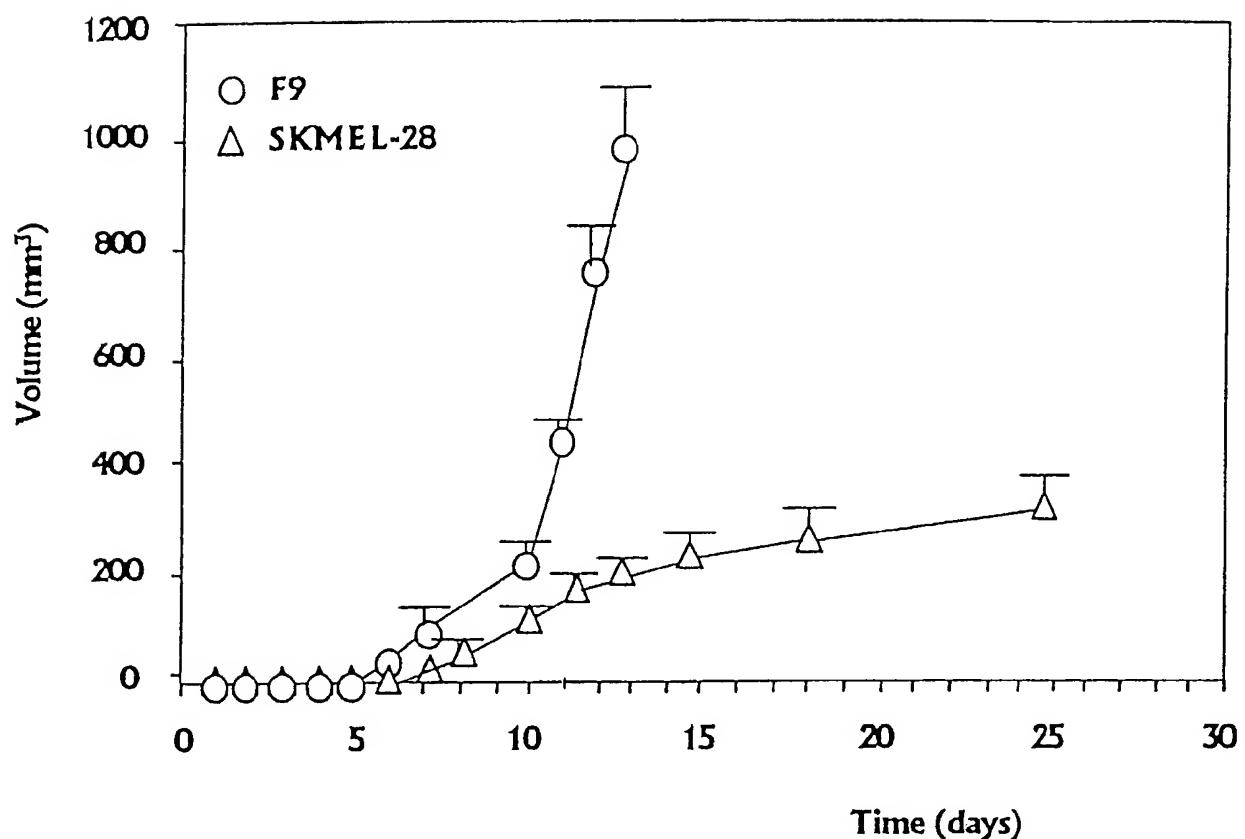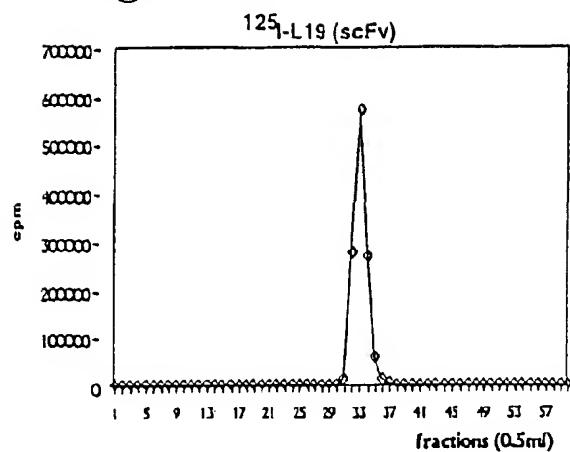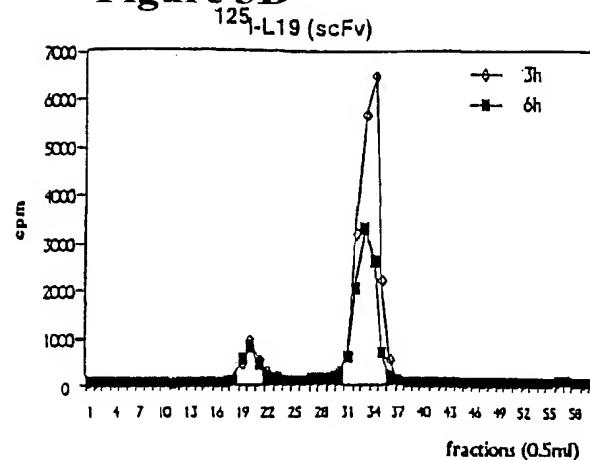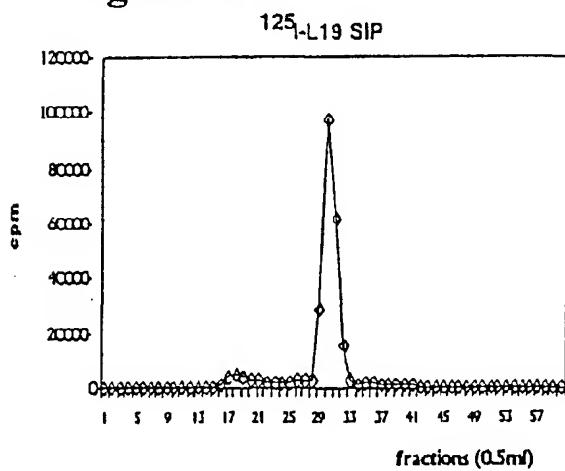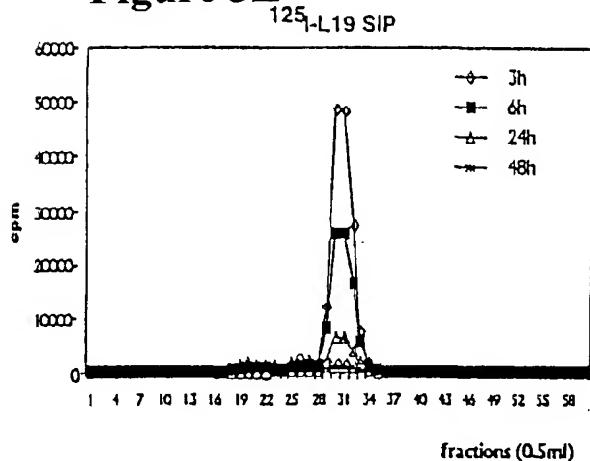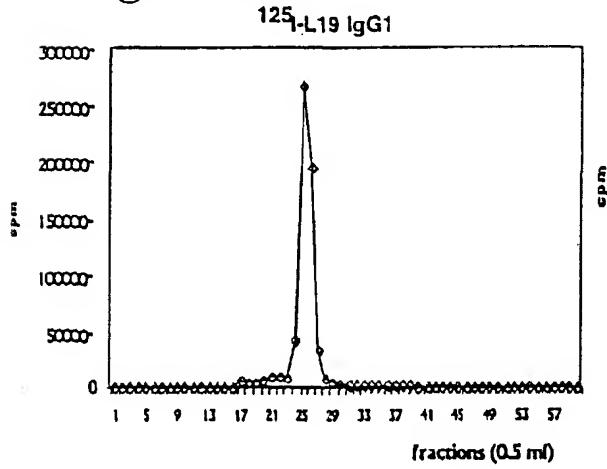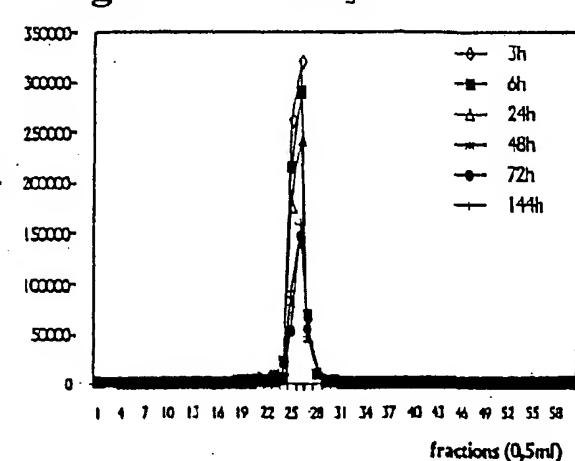
Figure 4A

Figure 4B

Figure 4C

Figure 5A

Figure 5B